DEVELOPMENTAL AND PHYSIOLOGICAL CHARACTERIZATION OF THE *MALE STERILE33* (*ms33*) MUTANT IN *ARABIDOPSIS*

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ABSTRACT

The objectives of this study were to characterize the *male sterile33* (*ms33*) mutant in *Arabidopsis thaliana* at the morphological and developmental levels, and to investigate the possible role of plant hormones in gene-controlled stamen and pollen development.

The morphology and growth of *ms33* and WT plants showed that the *ms33* mutation not only affects stamen and pollen development, but also has several pleiotropic effects including, aberrant growth of all floral organs, and delays in seed germination, seedling growth and flowering time. Ultrastructural studies showed that in anthers of the *ms33* mutant, there was premature degeneration of the tapetum which led to defects in intine maturation, tryphine deposition on the pollen wall, lipid body formation in the pollen, and the production of large, highly vacuolate non-viable pollen. These observations suggested that dehydration of pollen is impaired in the *ms33* mutant and that it affects pollen viability.

In the WT stamen, there was rapid growth of filaments before anthesis which was suppressed in the *ms33* mutant. This growth was mainly due to cell elongation and was stimulated by GA and IAA. The data suggest that the *MS33* gene controls filament growth by temporally stimulating GA and/or IAA biosynthesis, or hormone signal transduction pathways.

Seed germination was also delayed in the *ms33* mutant, but this was partially

overcome by low temperature and GAs. GA_4 was more effective than GA_3 in promoting seed germination, as well as seedling and plant growth. The *ms33* flowers contained relatively low levels of total GAs, in particularly GA_4, but a high level of GA_3. It is suggested that mutation in *MS33* favors the early 13-hydroxylation pathway of GA biosynthesis resulting in greater accumulation of GA_3, instead of the non-13-hydroxylation pathway in WT flowers that would lead to high GA_4 content. WT flowers also had a higher level of IAA, but lower level of ABA, than *ms33* flowers.

Analysis of double mutants of *ms33* with an ABA-deficient mutant *aba-1*, and a GA-signal transduction mutant *spy-3*, revealed that inhibition of filament growth and aberrant pollen development in *ms33* mutant are not related to high level of ABA or to a possible blockage in the GA signal transduction pathway.

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LIST OF ABBREVIATIONS

ABA	abscisic acid
aba-1	*abscisic acid-1*
BAP	benzylaminopurine
CMS	cytoplasmic male sterility
ER	endopasmic reticulum
EtOAc	ethyl acetate
GA(s)	gibberellin(s)
G-CMS	genic-cytoplasmic male sterility
GC-MS-SIM	gas chromatography-mass spectrometry-selected ion monitoring
GMS	genic male sterility
HAc	acetic acid
HPLC	high performance liquid chromatography
IAA	indole-3-acetic acid
LM	light microscopy
MeOH	methanol
ms33	*male sterile33*
PMC	pollen mother cell

PP333	paclobutrazol
RER	rough endoplasmic reticulum
SEM	scanning electron microscopy
spy-3	*spindly-3*
TEM	transmission electron microscopy
WT	wide type

I. INTRODUCTION

Sexual reproduction in angiosperms is a complex process that requires coordinated development of both the male and female reproductive organs. For male reproductive organogenesis, a number of developmental events are involved in both the sporophytic and gametophytic tissues. These include the initiation and differentiation of the stamen, i.e., filament and anther on the floral meristem, development of pollen grains including microsporogenesis and microgametogenesis inside the anther locules, their release from the anther, as well as pollen germination and pollen tube growth. Breakdown in any of these processes results in male sterility (Greyson, 1994). Male sterility in plants generally refers to the failure of an individual to produce functional anthers, pollen or male gametes under a given set of environmental conditions. However, its expression varies from the complete absence of stamens to the production of stamens with non-viable pollen or without pollen (Frankel and Galun, 1977; Kaul, 1988). The situation in which anthers contain viable pollen but fail to dehisce is generally defined as functional male sterility.

I.1. Male sterile systems and their value in breeding

The phenomenon of male sterility in bisexual flowering plants has been widely reported. Based on the genetic data from more than 600 species of monocots and dicots,

three classes of the inheritance patterns for male sterility are suggested (Frankel, 1973a; Kaul, 1988).

The genic male sterility (GMS), in most cases, is controlled by a single recessive nuclear gene, but can occasionally be also controlled by more than one recessive or by dominant gene (Athwal et al., 1967; Weaver, 1968; Weaver and Ashley, 1971; Mathias, 1985). The expression of sterility in GMS systems is entirely Mendelian.

Another type of male sterility, not under the influence of nuclear genes, is called cytoplasmic male sterility (CMS). These systems rely on cytoplasmic factors and genes in organelle, generally mitochondria, to affect the development of one or more tissues in the anther during some stage of microsporogenesis (Hanson and Folkerts, 1992; Williams and Levings, 1992; Levings, 1993; Hanson et al., 1995; Kempken and Pring, 1999). This type of sterility is maternally inherited. For some CMS systems there are male sterile nuclear genes (*fr*) that override the CMS condition. The *fr* gene action is cytoplasm-dependent. It is ineffective with normal (N) cytoplasm and acts only in sterile (S) cytoplasm. S-cytoplasm is ineffective in the presence of *Fr* nuclear genes. Thus, this male sterility is only caused by the interaction of nuclear genes (*fr*) and sterile (S) cytoplasm. These systems are designated as genic-cytoplasmic male sterility (G-CMS).

Although the phenomenon of male sterility in bisexual flowering plants was reported as early as the middle of the 18th century (reviewed in Frankel, 1973a), male sterility in higher plants is currently a popular subject of research. The potential use of male sterility for breeding cultivated plants is widely recognized and commercially utilized in the production of hybrid seeds (Driscoll, 1986; Kaul, 1988; Rao et al., 1990;

Horner and Palmer, 1995; Williams, 1995). Of the three types of male sterility, CMS and G-CMS are more commonly used in breeding due to their easy induction and maintenance (Frankel, 1973b). Very few, if any, hybrid seeds are presently produced with GMS systems as it is difficult to maintain pure male sterile lines due to recessive mutations in most GMS lines (Forsberg and Smith, 1980). GMS lines must be maintained by crossing with the heterozygotes and, thus, half of the offspring are fertile and have to be eliminated from the population (Frankel and Galun, 1977). However, the maintenance problem could be overcome via some approaches. For example, the one-half of fertile plants from the population may be isolated at an early stage of development by marker genes that closely link to the male sterile gene and affect vegetative features, e.g., seed and leaf shape and pigmentation (Driscoll, 1986; Rao et al., 1990). Restoration of male fertility in GMS lines by manipulations with chemicals (Hockett et al., 1978; Sawhney, 1984) and environmental factors (Sawhney, 1983a; Estrada et al., 1984) also has considerable potential in the production of hybrid seeds.

I.2. Genetic analysis of male sterility

Male reproductive organogenesis is a complex developmental process in which thousands of genes are expressed. Moreover, numerous genes expressed in the male gametophyte are also expressed in the sporophyte (Mascarenhas, 1990a). For example, 60% of the genes encoding different isozymes detected in vegetative tissues of tomato plants are also found in pollen (Tanksley et al., 1981), 72% in maize while only 6% of the isozymes are pollen-specific (Sari-Gorla et al., 1986). The total complexity of

tobacco anther mRNA has been estimated to be 3.23 x 10^7 nucleotides, equivalent to about 26,000 different genes (Kamalay and Goldberg, 1980). In the mature pollen of *Tradescantia*, there are more than 20,000 mRNA species which represent genes that are primarily transcribed late in pollen development, and about 10% of these genes are specific to pollen (Willing and Mascarenhas, 1984; Mascarenhas, 1990b; Schrauwen et al., 1990). These data underscore the complexity of the molecular processes leading to the development of reproductive organs. However, male sterile mutants may provide an important approach to identifying genes involved in the development of stamens and pollen.

Although a number of male sterile lines arise spontaneously, male sterility can be induced by, for example, radiation in *Chrysanthemum morifolium* (Broertjes and Jong, 1984) and chemical mutagens, such as ethyl methanesulfonate (EMS) in *Arabidopsis* (Veen and Wirtz, 1968) and copper chelators in wheat (Graham, 1986; Cross and Ladyman, 1991). Male sterility was also been generated by genetic engineering, such as fusion of protoplasts in tobacco (Kofer et al., 1990), transfer of a chimeric ribonuclease gene to tobacco and oilseed rape (Mariani et al., 1990), and transposon tagging of a male sterile gene in *Arabidopsis* (Aarts et al., 1993; Perison et al., 1997). A combination of gene transfer and chemical application was used, whereby a tapetum-specific deacetylase gene, which deacetylates externally-applied non-toxic *N*-acetyl-L-phosphinothricin to a compound that is toxic for the tapetal development, was induced in tobacco to induce sterility (Kreite et al., 1996).

A large number of male sterile mutants have been isolated from different plant

species. Based on the abnormalities, these mutants may be categorized into the following three types:

I.2.1. Structural male sterile mutants

Mutations that impair the formation of the male reproductive organ, but do not affect the female organ, are defined as structural male sterile mutants. Such mutants may completely lack the pollen-bearing organs (stamens) or their fertile parts (anther sacs). Often, the stamens formed are severely affected and pollen is rarely produced.

In a number of plants, including many crops, i.e., maize, soybean, rice and tomato, mutants have been described that alter the development of male structural organs. In the *stamenless-2* (*sl-2*) mutant of tomato, for example, the stamens do not interlock laterally and are shorter and paler in color than wild type (WT) stamens. Under normal conditions, the mutant anthers produce microspores, however, the majority either degenerate or are non-viable (Sawhney and Bhadula, 1988). The addition of GA_3 can restore pollen development in *sl-2* (Sawhney and Greyson, 1973). Several structural male sterile mutants have also been reported in maize. For example, the dwarf mutant *d2*, *d3*, *d5* and anther-ear mutants *an1* and *an2* possess smaller anthers than WT, and the anthers are devoid of pollen (Kaul, 1988).

In addition to the stamen phenotype, abnormalities in the carpel, petal and sepal whorls are also common in male sterile mutants, suggesting that some structural male sterile genes, such as homeotic genes, operate in the floral organ identity pathway (Coen, 1991). The homeotic mutation *pistillata* (*pi*) in *Arabidopsis* impairs whorls two

and three, generating a plant that is male-sterile but female-fertile (Bowman et al., 1989; Hill and Lord, 1989). In tomato, the most severe structural mutant, *pi* (*pistillate*) and *pi-2* (Rasmussen and Green, 1993) somewhat resemble homeotic mutants that have been described in *Arabidopsis*. Thus, *PI* is a structural male-fertile gene. In the mutant *deficiens* in *Antirrhinum majus*, female fertility is not impaired significantly, however, male organs are converted to abnormal female organs and petals are converted to sepaloid leaves (Sommer et al., 1990). The *deficiens* gene has been cloned and shown to encode a protein with a MADS box type DNA binding motif. The motif has been identified in other plant organ identity genes (Coen and Meyerowitz, 1991). In the *antherless* (*at*) mutant in *Arabidopsis*, filaments are present but anther lobes are either not fully differentiated or are converted to sepals (Chaudhury et al., 1992), indicating that this gene is required not only for anther formation but also for other floral organs.

The studies on structural mutants do not provide specific information on pollen development *per se*, but may suggest the gene interactions that regulate the formation of cell types critical for pollen development.

I.2.2. Sporogenous male sterile mutants

Sporogenous mutants are essentially morphologically similar to WT, but they differ from fertile plants in their ability to produce functional pollen. Analyses of these mutants may provide information on the sporophytic genes required for pollen development. A number of sporogenous male sterile mutants have been described from a variety of species. These mutations may affect different stages during pollen

development, i.e., premeiotic, meiotic, tetrad, microspore and post-microspore stages. Grouping the mutants according to the stage of pollen abortion may indicate which genes act at different stages of the pollen developmental pathway.

The mutants that are impaired in microspore development can be separated into two broad categories: 1) those in which microspore tetrads are aberrant, indicating a premeiotic or meiotic defect; 2) those in which normal meiosis occurs to produce microspore tetrads, defects at a later stage of pollen development.

In tomato mutants *ms3* (Andrasfalvy, 1970), *ms15* (Rick and Zischke, 1987), and *ms29* and *ms32* (Stevens and Rick, 1986), meiosis does not occur and mostly degenerated pollen mother cells (PMCs) are formed. In another large group of tomato mutants *ms-1*, *ms-5*, *ms-7*, *ms-8*, *ms-10*, *ms-12*, *ms-16*, *ms-18*, *ms-30* and *ms-33*, abortion occurs during meiosis (Gorman and McCormick, 1997). These mutants identify the genes that control premeiotic or meiotic sporogenesis, and the differentiation of archesporial tissues. In maize, the mutants *ms8* and *ms9* have abnormal PMCs which are small and irregular in form, and degenerate quickly (Albertsen and Philips, 1981). Maize *ms17* mutant has defects including excess microtubules, abnormal spindle formation and improper chromosome segregation during meiosis (Staiger and Cande, 1991). A complete failure of cytokinesis after telophase II in soybean male sterile line N69-2774 results in large size of multinucleate pollen grains which are nonfunctional (Patil and Singh, 1976). Similarly, the absence of, incomplete or disoriented cytokinesis following telophase II gives rise to cells with different numbers of nuclei in soybean *ms4* mutant (Graybosch and Palmer, 1985b).

Semmes M.S.-2 line of soybean has normal cytokinesis, but nuclei degenerate immediately after that and no microspores are produced (Patil and Singh, 1976). Sterility in a newly isolated male sterile mutant *msMOS* in soybean is caused by failure of callose dissolution at the tetrad stage (Jin et al., 1997). Numerous mutants in which abortion of microsporogenesis occurs during the tetrad stage have also been isolated in tomato, such as *ms-2, ms-17, ms-23, ms-34, ms-45* and *ms-46*. In these mutants complete normal meiosis occurs before breakdown of microsporogenesis (Gorman and McCormick, 1997). In *Arabidopsis* mutants *ms3*, *ms4*, *ms5*, *ms15* (Chaudhury et al., 1992) and *ms32* and *ms37* (Dawson et al., 1993), the sporogenous tissue is normal while tetrads are aberrant. In the cytokinesis mutant *std* or *stud* isolated and characterized from *Arabidopsis* (Hülskamp et al., 1997), although the male meiotic nuclear divisions are normal, no cell walls are formed resulting in tetranucleate microspores, indicating that the *STD* gene is specifically required for male-specific cytokinesis after telophase II.

Many male sterile mutants produce normal tetrads. In these cases, sterility is a manifestation of a post-meiotic event. In the *qrt1* and *qrt2* mutants of *Arabidopsis*, normal tetrads are formed, but microspores are not released because pectin in the PMC wall persists after degradation of the callose wall, indicating that *QRT1* and *QRT2* may be required for cell type-specific pectin degradation for microspore separation (Rhee and Somerville, 1998). In the *ms1* mutant of *Arabidopsis*, microspores are released from normal tetrads, but subsequently develop an abnormal vacuolated appearance (Chaudhury et al., 1992). In seven other male sterile mutants (*ms8* - *ms13*) isolated from *Arabidopsis* abnormalities at post-meiotic stages are also shown (Taylor et al., 1998). In

maize, seven male sterile mutants with abnormal development after the tetrad stage have been reported (Albertsen and Philips, 1981). In the *ms7* mutant, microsporogenesis is normal until tetrad stage; thereafter, chromosomes become precociously condensed. In *ms10* and *ms13* mutants, thickened microspore walls develop after they are released from tetrads. But in the *ms12* mutant, microspore wall is normal and nuclear development is arrested, indicating that gametophyte nucleus is not involved in exine formation. Microspore development in *ms5*, *ms11* and *ms14* is arrested at mitotic stage. In *gametophytic male sterile-1* (*gaMS-1*) mutant in maize, identified by transposon insertion mutagenesis (Sari-Gorla et al., 1996), *gaMS-1* expresses soon after the first mitosis of microspores and leads to the production of immature, non-functional pollen grains. In a soybean male sterile line Semmes M.S.-1, cytokinesis is normal, but pollen development is defective resulting in nonfunctional pollen grains (Patil and Singh, 1976). In tomato mutants *ms-9*, *ms-13*, *ms-14*, *ms-24*, *ms-27*, *ms-28*, *ms-31*, *ms-37*, *ms-41*, and *ms-43*, pollen development aborts after the formation of free microspores (Gorman and McCormick, 1997).

I.2.3 Functional male sterile mutants

Functional mutants produce viable pollen but have defects in anther structure that prevent effective pollen release, implying that the release of pollen from the mature anther is also under genetic control. These mutants provide information about the mechanisms governing the process of self-fertilization, i.e., how pollen is directed to the stigma. Relatively few mutants displaying functional male sterility have been described.

A functional mutant *dialytic* (*dl*) has been reported in tomato (Rick, 1947). This mutant prevents the growth of epidermal hairs on the anther surface. The anthers fail to hold together around the pistil and the pollen grains are not directed toward the style and rarely reach the stigma. In the *Arabidopsis* mutant *ms35*, functional pollen grains inside the anther locules are not released due to a failure of anther dehiscence (Dawson et al., 1993). Mutations that inhibit anther dehiscence have also been reported in barley (Roath and Hockett, 1971) and tomato (Roever, 1948). These genes may affect aspects of anther wall anatomy required for the rupture and opening of the stomium after pollen maturation. Stomium rupture is the ultimate event essential for anther dehiscence (Goldberg et al., 1993). Recently, the *barnase* and *barstar* genes have been fused to promoters with different cell specificities, and transferred into tobacco plants to ablate either the stomium and the circular cell cluster or the stomium region alone. The results demonstrate that a set of functional stomium cells are required for anther dehiscence and pollen release (Beals and Goldberg, 1997).

I.2.4. Mutants defective in tapetum

Numerous male sterile mutants in both CMS and GMS systems exhibit different abnormalities in the ontogeny of the tapetal layer. In many cases, the defects in the tapetum are observed prior to or at the time of breakdown of microsporogenesis and pollen maturation. Thus, abortion of pollen development is often attributed to the aberrant development of the tapetum (Edwardson, 1970; Bhandari, 1984). For example, in CMS sorghum, the tapetum enlarges with a vacuolate cytoplasm (Overman and

Warmke, 1972). The tapetum of CMS sunflower encroached upon the locule at the tetrad stage (Horner, 1977), and similarly, the tapetum often becomes highly vacuolated at the early tetrad stage in the C-CMS line of maize (Lee et al., 1979). In GMS lines, e.g. soybean *ms3* mutant, premature degeneration of tapetal tissue results in the formation of unusually enlarged microspores (Palmer et al., 1980; Nakashima et al., 1984; Graybosch and Palmer, 1987). Similarly, premature vacuolation of tapetal tissue causes microspore degeneration after the deposition of primexine and probacullae in the *ms2* mutant in soybean (Graybosch and Palmer, 1985a) and abortion of reproductive cells at all stages of anther development in the *msp* mutant (Stelly and Palmer, 1982). Young microspores of the maize mutant *ms25* vacuolate and degenerate due to accumulation of large lipid bodies and large vacuoles in tapetal cells at this stage. Similarly, abortion of young microspore development in the *ms26* mutant is attributed to vacuolation of tapetal cells (Loukides et al., 1995). The premature breakdown of the tapetum is also found in the tomato *ms11 mutant* (Gorman and McCormick, 1997). On the other hand, the tapetal breakdown is delayed in tomato mutants *ms6*, *ms7*, *ms8*, *ms10*, *ms13*, and *sl-2* (Gorman and McCormick, 1997; Polowick and Sawhney, 1995). In each of these mutants both the tapetal cells and the sporogenous tissues are affected. In *Arabidopsis*, the early degeneration of tapetum leads to breakdown of meiosis in the *ms32* mutant (Dawson et al., 1993; Fei and Sawhney, 1999) and degeneration of microspores in *ms7* (Taylor et al., 1998). These observations indicate that the tapetum plays an important role in pollen development.

I.3. Environmental factors and male sterility

The expression of male sterility in many GMS and CMS plants and in fertile plants is affected by environmental factors. The major factors are temperature, photoperiod and water stress. The interactions between plants and the environment vary with the genotype.

I.3.1. Temperature

Temperature is a potent external factor that affects the expression of male sterility. However, the effects of temperatures on the restoration of male fertility are variable and diverse in different male sterile systems. In *sl-2*, a GMS mutant, low temperatures (18/15°C day/night) induce fertility, but carpeloid stamens are produced at high temperatures (28/23°C) (Sawhney, 1983a) . A similar result was reported in a partial male sterile soybean mutant (*msp*) subjected to low night temperatures (Carlson and Williams, 1985). Low temperature also favors the restoration of male fertility more than the normal growing conditions in CMS wheat (Johnson and Patterson, 1973) and maize (Duvick, 1965). A comparative study on the effect of different temperatures on male fertile, partially-restored and fully-restored lines of petunia shows that all the lines produce fully-fertile anthers at low temperatures (17-18/15 °C), but both restored lines are fully sterile; the fertile line is not affected at high temperature (35/15°C) (Izhar,1975). In contrast, in G-CMS systems of beet (Cleij, 1967), rye (Scoles and Evans, 1979), and sorghum (Zhang and Fu, 1982), the expression of male sterility is increased with low temperatures, which is reduced following high temperature

treatments. Moreover, a CMS mutant *pol* in *Brassica napus* is partially restored at high temperature (30/24 °C) and the *nap* mutant becomes fully fertile under similar temperatures (Fan and Stefansson, 1986).

Even in normal fertile plants, there is much variation in pollen fertility when exposed to different temperatures, e.g. high temperature reduced pollen fertility in cultivars of spring wheat (Welsh and Klatt, 1971). In rice and sorghum, however, treatment with low temperature at meiotic stage leads to pollen sterility (Ito, 1978; Brooking, 1979).

The mechanism of temperature effect on the expression of male sterility is still unclear. These differential responses are considered to be related to different genes that act at different stages (Kaul, 1988).

I.3.2. Photoperiod

In many angiosperms, flowering is induced by photoperiod or by low temperature (vernalization). The influence of photoperiod on the expression of male sterility has been reported in a number of male sterile mutants. A photosensitive barley mutant ms_9 exhibits a fully male sterile condition under long days in Finland, but is partially sterile under relatively short photoperiod in Bozeman, USA (Ahokas and Hockett, 1977). Similarly, wheat with *Aegilops crassa* cytoplasm is almost completely male sterile when grown under long day conditions, but is highly male fertile under short day (Murai and Tsunewaki, 1993).

A recessive male-sterile rice also exhibits photoperiod-sensitivity. It is male

sterile under long days (exceeding 13.5 h) and fertile in short days (Shi, 1985). Further studies indicate that alteration of male fertility is photoperiod-sensitive only at the phase from secondary branch primordia differentiation to pollen mother cell formation (Yuan et al., 1993). Investigation on the mechanisms of phytochrome action in this mutant suggests that the photoperiod response affecting fertility alteration is different from that affecting flowering, i.e., fertility alteration and flowering may be controlled by separate phytochrome signaling pathways (Wang, 1998).

Some temperature-sensitive mutants, e.g. pepper, tomato (Martin and Crawford, 1951) and sesame (Brar, 1982), also respond to photoperiod. Transfer of these mutants from glasshouse to field or *vice versa* can induce fertility or sterility in them.

I.3.3. Water stress

Pollen development is highly sensitive to water stress, especially at the stages from meiosis to microspore release (Saini, 1997). Male sterility induced by water stress has been reported in some species. In wheat and rice, male reproductive development is very sensitive to water stress. Pollen sterility can be induced by a short period of drought during meiosis (Morgan, 1980; Saini and Aspinall, 1981; Sheoran and Saini, 1996). Male sterility characterized by the production of small flowers with dysfunctional stamens is also induced by drought in an island plant, *Cedronella canariensis* (Olesen et al., 1998). Cytological studies have shown that sterile pollen caused by water stress lack starch (Sheoran and Saini, 1996). Water stress also changes the allocation of starch in the anthers (Lalonde et al., 1997). It has been suggested that

male sterility induced by water stress may be associated with carbohydrate availability and/or metabolism during pollen development (Saini, 1997).

I.4. Role of plant hormones in male sterility

It is well known that plant hormones have a role in the regulation of nearly every aspect of plant growth and development (Davies, 1988). The alterations in the expression of flower development are often accompanied by alterations in the plant hormone status in the floral meristem and associated tissues (Chailakhyan and Khrianin, 1987; Metzger, 1988). The data obtained from exogenous hormonal treatments and analyses of endogenous hormones in a number of male sterile systems and normal plants show that nearly all types of hormones are directly or indirectly involved in stamen and pollen development (Sawhney and Shukla, 1994). However, whether the expression of male sterility is regulated by plant hormones is not clearly understood.

I.4.1. Cytokinins

Cytokinins have been implicated specifically in the development of female reproductive organs, e.g. in *Mercurialis annua*, cytokinins induce pistillate flowers on male plants (Durand and Durand, 1991). It has been reported that cytokinins are involved in the expression of cytoplasmic male sterility in barley (Ahokas, 1982). The lowest quantity of endogenous cytokinins occur in the unrestored male sterile lines, whereas in the restored line and the fertile line the cytokinin levels are high and similar. In the CMS mutant *ogu* of *B. napus*, male sterility is partially related to a deficiency of

active cytokinins, especially dihydrozeatin (Singh and Sawhney, 1992). Analysis of endogenous cytokinins in a GMS line of *Brassica* show that although there is a high level of dihydrozeatin in the leaves, it is low in the flowers and stamens of male sterile plants, in comparison to WT (Shukla and Sawhney, 1992). However, it has also been reported that some male sterile plants have higher levels of cytokinins than do male fertile plants (Musgrave et al., 1986). Similarly, male sterile and restored fertile strains of *M. annua* have higher levels of *cis*-zeatin and *cis* zeatin riboside in shoot apices than normal fertile male lines (Louis et al., 1990). Thus, it is evident that the types of cytokinins and their levels vary in different male sterile systems.

Three adenine phosphoribosyl transferase (APRT)-deficient mutants (BM1~3) isolated from *A. thaliana* are male sterile due to abortion of pollen development after meiosis (Moffatt and Somerville, 1988). Further investigation shows that the metabolism of cytokinins is impaired in the BM3 mutant and the levels of cytokinin nucleotides is low (Regan and Moffat, 1990; Moffatt et al., 1991). The results suggest that the expression of male sterility in these mutants may be associated with the altered metabolism of cytokinins.

I.4.2. Gibberellins

A number of experiments on exogenous gibberellins (GAs) suggest that GAs have a role in normal stamen and pollen development. For example the number of staminate flowers in a gynoecious cucumber line is increased following GA application (Pike and Peterson, 1969) and in some GMS mutants, GAs restore stamen development

and male fertility, e.g. *stamenless* tomato mutants (Phatak et al., 1966; Sawhney and Greyson, 1973) and barley male sterile mutant (Kasembe, 1967). Strong evidence for the involvement of GAs in the expression of male sterility comes from some GA-deficient mutants. For example, in tomato *ga-1* and *ga-2* mutants, in which stamen and pollen development is impaired, application of GAs restores male fertility (Nester and Zeevaart, 1988; Jacobsen and Olszeweski, 1991).

The analyses of endogenous GAs in male sterile and normal fertile plants show that male sterile lines generally have lower levels of GAs than do the WT. The male sterile *sl-2* mutant of tomato contains lower levels of GAs in vegetative parts and flowers than those in WT (Sawhney, 1974). Similarly, in a GMS line of rice, the levels of GA_1 and GA_4 in the anthers are about one-fifth to one-sixth of those in the fertile line (Nakajima et al., 1991). The possible involvement of endogenous GAs in the expression of male sterility is supported by the experiments with inhibitors of GA biosynthesis. For example, 2-chloroethyl-trimethyl ammonium chloride (CCC) prevents the GA-induced restoration of male fertility in the tomato *sl-1* mutant (Phatak et al., 1966). The involvement of GAs in the expression of male sterility is also confirmed by gene cloning. For example, in maize anther-ear mutants *an1*, the *AN1* gene has been cloned and characterized. Its product is involved in the synthesis of *ent*-kaurene in GA biosynthetic pathway (Bensen et al., 1995).

However, in some systems GA application is ineffective in restoring fertility in male sterile mutants. In *in vitro* culture of floral meristems of a CMS tobacco mutant (Hicks et al., 1981), and culture of tassels of maize *ms14* and *ms24* mutants, the

presence of GAs does not promote pollen development (Pareddy, 1990). In flower culture of *sl-2* mutant of tomato, floral organs do not develop in the medium lacking GA, but in the presence of GA, well-developed flowers are produced; however, they are male sterile (Rastogi and Sawhney, 1988). These results indicate that other factors in addition to GAs may be required for normal flower and pollen development.

I.4.3. Auxins

It has long been known that auxins promote the formation of female organs and suppress the development of male organs (Chailakhyan and Khrianin, 1978, 1987). Application of 2,4-dichlorophenoxyacetic acid (2,4-D) induces pollen sterility in tomato (Rehm, 1952), and indole-3-acetic acid (IAA) induces the carpellization of stamens in the *sl-2* mutant of tomato (Sawhney and Greyson, 1973). Analyses of endogenous IAA in different male sterile systems have shown that the levels of IAA in male sterile lines are generally higher compared with the normal fertile plants. In male sterile *M. annua*, auxin quantities are higher than in the normal males (Durand and Durand, 1991). Similarly, the levels of IAA in the leaves and stamens of *sl-2* mutant grown in high temperatures are greater than WT, but are similar in low temperature, when fertility is restored in the mutant (Singh et al., 1992). In transgenic tobacco plant, *rolB* gene causes an increase in free IAA level and this plant exhibits abnormal development of anther and pollen (Estruch et al., 1991). In contrast, in the various male sterile lines of *M. annua*, the level of IAA in apices is low in male sterile line, but increases in semisterile and restored fertile lines (Louis et al., 1990). In a CMS line in rice, the levels of IAA in

leaves, anthers and panicles are lower than those in the maintainer line, but abscisic acid (ABA) is higher (Tian et al., 1998), further indicating that other growth factors may be involved in the expression of male sterility.

I.4.4. ABA and ethylene

ABA and ethylene are generally considered as inhibitors of plant growth and are known to affect pollen development and induce male sterility in a number of species including wheat (Hughes et al., 1978; Morgan, 1980; Keyes and Sorrells, 1990), barley (Colhoun and Steer, 1983), millet (Thakur and Rao, 1988), *Brassica* (Banga and Labana, 1983) and tomato (Chandra Sekhar and Sawhney, 1991). In the *sl-2* mutant, the levels of ABA in vegetative and floral parts are higher than those in WT, especially in stamens. Low temperatures restore male fertility in *sl-2* and reduce the level of ABA (Singh and Sawhney, 1998), indicating possible involvement of a high level of ABA in the expression of male sterility in the *sl-2* mutant. An ABA-deficient mutant *aba-1* in *Arabidopsis* is also male fertile (Koornneef et al., 1982), suggesting that reduced ABA level does not induce the expression of male sterility. Previous studies show that the expression of male sterility caused by water stress is related to high ABA levels (Morgan, 1980; Saini and Aspinall, 1981), However, recent research suggests that stress-induced abortion of pollen development is preceded by disturbances in carbohydrate metabolism and distribution within anthers, and not by ABA (Saini, 1997).

A male sterile mutant of rice, which is photoperiod and temperature sensitive, increases ethylene biosynthesis after treatment with 1-amino-cyclopropane-1-carboxylic

acid (ACC), and the level of fertility decreases sharply. If plants are treated with amonothoxy vinylglycine (an inhibitor of ethylene biosynthesis), male fertility is increased (Li et al., 1996). These results indicate that ethylene release is negatively correlated with male fertility. However, genetic transformation in tobacco shows that both *alpha-AIB* and the *rolC* genes reduce ethylene production and cause male sterility (Martin et al., 1993).

I.5. Objectives of this study

From the review above it is obvious that in many cases the expression of male sterility can be affected by internal and external factors, i.e., endogenous plant hormones, exogenous hormones, and environmental factors. However, it is not clear whether plant hormones are directly or indirectly involved in the expression of male sterility, and whether plant hormone-mediated induction or reversion of male sterility is influenced by the environment.

Arabidopsis thaliana (L.) Heynh. Var. Landsberg *erecta* is a small annual weed of the mustard or crucifer family (Brassicaceae). It has a short life cycle and small genome size (Meyerowitz, 1987) and has been widely used as a model system for plant molecular, genetic, developmental, physiological and biochemical studies. A number of floral mutants have been genetically well characterized. *Male sterile33* (*ms33*) mutant, formerly known as *msZ*, was isolated by EMS mutagenesis (Dawson et al., 1993). The inheritance of the mutant is controlled by monogenic recessive mutation. This mutant has a defect in both stamen filament elongation and pollen maturation. Anthers of *ms33*

show abnormalities in tapetal cells, anther dehiscence is delayed, and pollen produced are non-viable (Dawson et al., 1993). However, cytological and physiological changes during pollen development, the effects of environmental factors on the expression of male sterility, and the mechanism of control of filament growth in this mutant have not been examined.

The overall objective of the present study was to characterize the development of stamen and pollen in *ms33* mutant and to investigate the possible role(s) of plant hormones in the expression of male sterility in this mutant. The specific objectives, and experimental approaches used in this study, were as follows:

1. To describe the morphology and growth of *ms33* and WT plants and their flowers.

2. To examine the cytological changes in pollen development of *ms33* and WT anthers by light microscopy (LM), scanning electron microscopy (SEM) and transmission electron microscopy (TEM).

3. To investigate the growth of *ms33* and WT stamen filaments *in vivo* and *in vitro* and to determine the role of anther and hormones in filament growth.

4. To determine the effects of environmental factors on stamen and pollen development in *ms33* mutant.

5. To test the pleiotropic effects of mutation in *MS33* gene on seed germination and plant growth.

6. To analyze the status of endogenous plant hormones in mature flowers of *ms33* and WT plants grown under normal and low temperatures.

7. To construct double mutants by combination of *ms33* and *aba-1* (ABA-deficient mutant), and *ms33* and *spy-3* (a GA signal transduction mutant), respectively.

II. MATERIAL AND METHODS

II.1. Plant material and growth conditions

Seeds of WT *Arabidopsis thaliana* Landsberg *erecta* and the *ms33* mutant, produced by ethyl methane sulfonate (EMS) mutagenesis (Dawson et al.,1993), were provided by Dr. B. Mulligan of the University of Nottingham, U.K. Seeds of the *spy-3* mutant (Columbia ecotype) were obtained from the Arabidopsis Biological Research Centre, Ohio State University, Columbus, OH, U.S.A., and of the *aba-1* mutant of (Landsberg *erecta* ecotype) from the Nottingham Arabidopsis Stock Centre, U.K. Seeds were sown in 15 cm plastic pots containing Tera-lite Redi-earth mix (W.R.Grace and Co., Ajax, Ontario, Canada) and exposed to 4°C in the dark for 3 days. The pots were then transferred to a growth chamber set at 22/18°C (day/night) temperature and 16/8 h photoperiod. The pots were placed in large trays and watered from below. Fluorescent tubes provided the light source (Osram Sylvania Ltd., Versailles, KY, U.S.A.) at 120-150 μmol m^{-2} s^{-1}.

The original *ms33* seed was F_2 and produced a 3:1 ratio of male fertile to male sterile plants. A back cross of *ms33* was made with the heterozygote and the seeds produced a 1:1 ratio of male fertile and male sterile plants.

II.2. Growth measurements

The height of both the *ms33* and WT plants, mainly contributed by peduncles, was measured after 6 weeks when WT plants had stopped flowering. Thirty plants of each genotype were measured. Sepals, petals, stamens and carpels of both the *ms33* mutant and WT flowers were excised at anthesis, and the lengths of floral organs (from 20 flowers) were measured using a Nikon SMZ-10 stereo dissecting microscope. Filament lengths of both the *ms33* and WT long stamens were measured from floral buds of different developmental stages, and from flowers at anthesis. Measurements of epidermal cells of filaments were made by preparing squashes of mid-regions of filaments and by examining tissue segments with a Nikon Optiphot compound microscope. For filament growth, one long stamen from each of 20 floral buds at different developmental stages and at anthesis, was measured. For epidermal cell length, a minimum of 200 cells in the mid-region of filament, randomly selected from 20 long stamen filaments from different flowers, was measured.

II.3. Microscopy

II.3.1. Light microscopy

II.3.1.1. Fixation of material

Floral buds of different sizes were fixed in 1.5% glutaraldehyde and 1% paraformaldehyde in 0.025 M phosphate buffer (pH 7.0) for 1 h at room temperature, and then transferred to 3% glutaraldehyde for 2 h, and postfixed in 1% osmium tetroxide overnight. The samples were dehydrated in a graded ethanol series for 15 min

per 10% increment, on ice. The dehydrated samples were infiltrated with propylene oxide by dropwise addition over a period of 8 h on ice. The material was stored overnight in 100% propylene oxide in a freezer. One-half of propylene oxide was poured off and replaced with 50% Araldite (502) resin (diluted in propylene), by drop wise addition, over a period of 8 h at room temperature. One-half of the diluted resin was removed from the vial, and 100% resin was added by the same method as above. The remaining propylene oxide was allowed to evaporate in a fume hood overnight. Each floral bud was embedded in a small aluminum tray containing fresh resin and the tray kept in an oven at 60°C for 2 days to solidify the resin.

II.3.1.2. Sectioning and staining

Different sizes of floral buds in the solidified resin were trimmed with a small coping saw. Each bud was mounted onto a resin block with 5 min epoxy resin and sectioned on a microtome (Reichert-Jung, Ultracut, Austria) with a glass knife. For LM, the sections (approx. 1 μm in thickness) were mounted in a drop of water on a glass slide and gently heated to dryness over an alcohol lamp. Sections were stained with 1% toluidine blue (in 1% borax) and warmed gently. The stain was rinsed off with distilled water and the slides dried on a heating plate (Chicago Surgical and Electrical Co., U.S.A.) set at 40°C. A drop of mounting medium (Cytoseal 60) was put on the sections and covered with a coverslip (No. 2, VWR Scientific Inc.). The slides were viewed through a Nikon Optiphot compound microscope with an attached Nikon Microflex camera. For photography, Kodak T-Max (ASA100) film was used and developed with

Kodak HC110 (1:9) developer.

II.3.2. Scanning electron microscopy

II.3.2.1. Fixation and critical-point drying of samples

Young inflorescences and mature flowers were fixed in 3% glutaraldehyde in 0.025 M phosphate buffer (pH 7.0) at room temperature overnight. The material was rinsed with the same buffer, postfixed in 1% osmium tetroxide (in the same buffer) for 2 h, rinsed again with distilled water, and dehydrated on ice with a graded acetone series (15 min each step) in a 10% increment. The samples were stored in fresh 100% acetone overnight in a freezer, and then dried with liquid CO_2 in a critical point dryer (Polaron, Watford, England). The acetone was replaced by liquid CO_2 two times with 1 h interval between them. Finally, the dryer was heated to 30°C, and CO_2 was released slowly from it.

II.3.2.2. Coating and scanning

The dehydrated samples were mounted on aluminum SEM stubs with double sided tape and coated with gold in a Edwards Sputter Coater (S150B, England) for 3 min. The coated specimens were observed in a Philips 505 scanning electron microscope at an accelerating voltage of 30 kV. The images were photographed on Polaroid 665 P/N film.

II.3.3. Transmission electron microscopy

II.3.3.1. Fixation of material

The material and fixation procedures used for TEM were the same as those for LM (II.3.1.1).

II.3.3.2. Sectioning and staining

The sample blocks were initially cut with a glass knife as for LM, and the sections were examined to select different stages of pollen development. The blocks were then cut with a diamond knife (Institute Venezolano de Investigacions Cientificas, Venezuela) on the same microtome, and gray to gold sections were picked up on 100-mesh copper grids coated with Formvar (0.5% in ethylene dichloride). The sections were stained with uranyl acetate (a saturated solution in 70% ethanol) and Reynolds' lead citrate stain (Reynolds, 1963) in the dark for 30 min and 10 min, respectively. The grids were gently rinsed with distilled water after each stain and put on silicone rubber mats (J.B. EM. Services, Montreal, QC, Canada) in Petri dishes to dry naturally.

The sections were viewed through a Philips CM 10 transmission electron microscope at 80 kV and the images photographed on Kodak 4489 EM film. The film was developed with Kodak D19 developer for 4 min.

II.3.4. Sources of chemicals used in microscopy

Sources of chemicals used in microscopy are listed in Table 1.

Table 1. Source of the chemicals and their sources used in microscopy

Chemical	Source
Araldite 502 resin	J.B. EM Services Inc.
Dibasic sodium phosphate	Fisher Scientific
Dodecenyl succinic anhydride	J.B. EM Services
Ethylene dichloride	JBS-CHEM
Formvar 15/95 powder	J.B. EM Services Inc.
Glutaraldehyde	"
Lead nitrate	Fisher Scientific
5-minute epoxy resin	Devcon Corp.
Monobasic sodium phosphate	Fisher Scientific
Mounting medium	Stephens Scientific
Osmium tetroxide	J.B. EM Services
Paraformaldehyde	"
Propylene oxide	J.B. EM Services Inc.
Sodium citrate	Fisher Scientific
Tetrasodium borate (borax)	"
Toluidine blue	"
Tri-(dimethylaminomethyl)phenol (DMP-30)	J.B. EM Services Inc.
Uranyl acetate	"

II.4. Pollen germination *in vitro*

WT and *ms33* mature pollen grains were germinated in the Hodgkin and Lyon's (1986) medium [100 mg/l H_3BO_3; 399 mg/l $Ca(NO_3)_2 \cdot 4H_2O$; 100 mg/l KNO_3; 207 mg/l $MgSO_4 \cdot 7H_2O$; 4.865 g/l *N*-Tris(hydroxymethyl-methyl-3-amino-propane- sulphonic acid (TAPS)] using a sitting drop culture method (Shivanna and Rangaswamy, 1993). One drop (ca. 50 µl) of the culture medium was placed on a clean, dry microscope slide. Pollen grains were homogeneously distributed in the drop. The culture was kept across two supporting glass rods in the improvised humidity chamber at 22°C for 5 h. At the end of culture, a drop of fixative (10% ethanol) was added in the medium. A cover glass was lowered and germination was examined under a compound microscope. Three slides were prepared for each genotype.

II.5. Exogenous plant hormones

II.5.1. Foliar spray application

WT and *ms33* plants were treated with H_2O (control), 10^{-3}, 10^{-4} or 10^{-5} M ABA, IAA, GA_1, GA_3, zeatin or N^6-benzylaminopurine (BAP) (for sources, see Table 2) by foliar spray 1 week before flowering. Tween-20 (polyoxyethylene-sorbitan monolaurate, BDH Chemicals) (0.02%, v/v) was added into each hormone solution as a surfactant. The foliar spray was applied until the solution runoff. Each application was made four times at a 3-day interval. At least 40 plants were sprayed for each treatment.

Table 2. Sources of plant hormones and growth regulating substances used in this study.

Chemical	Source
Abscisic acid (ABA)	Sigma Chemical
N6-benzylaminopurine (BAP)	"
Ethrel	Union Carbide
Gibberellic acid (GA_3)	Sigma Chemical
Gibberellin A_4 (GA_4)	Dr. R. Pharis, University of Calgary
Indole-3-acetic acid (IAA)	Sigma Chemical
Paclobutrazol (PP333)	Dr. R. Pharis, University of Calgary
Zeatin	Sigma Chemical

II.5.2. Microdroplet application

Microdrops, i.e., 2, 5 or 10 μmol of one of ABA, IAA, GA_1, GA_3, zeatin, N^6-benzylaminopurine (BAP) solution or equal volume of H_2O (control) were loaded onto the secondary inflorescences (containing approximately 10 floral buds) of the mutant and WT plants by a microsyringe. The inflorescences were treated only once. Each treatment consisted of at least 20 inflorescences on different plants. All hormone solutions contained 0.02% (v/v) Tween-20 as a surfactant.

The effects of hormones on plant growth, and stamen and pollen development in both the genotypes, were examined. The lengths of floral organs from 20 flowers and the

lengths of 200 epidermal cells from 20 stamen filaments from different flowers were measured.

II.6. Environmental treatments

II.6.1. Photoperiod

After seed germination, WT and *ms33* mutant plants were grown in three different photoperiods, i.e., 8/16, 16/8 and 20/4 h (day/night) in growth chambers (ER 731, Enconaire Systems Ltd., Winnipeg, Canada). Light source was fluorescent tubes (Osram Sylvania Ltd., Versailles, KY, U.S.A.) at 120 μmol m^{-2} s^{-1}. Temperature was set at 22/18°C (day/night) for all photoperiods. For each treatment, at least 150 plants of each genotype were used. The effects of photoperiods on plant growth, and on stamen and pollen development, were examined by microscopy in both genotypes.

II.6.2. Temperature

The F_2 seeds of *ms33* were sown as described in II.1. All fertile plants in the F_2 population were removed based on stamen morphology in the first flower. WT and male sterile plants were grown in five different temperature regimes, i.e., 12/10°, 15/11°, 18/15°, 30/24° and 22/18°C (control) day/night. Photoperiod was set at 16/8 h day/night. The light source and intensity were the same as in II.1. At least 150 plants were grown in each temperature regime. The effects of temperature on the growth of floral organs, especially stamens, and pollen viability in both WT and *ms33* plants, were determined.

II.7. Emasculation of flowers and treatments

II.7.1. *in vivo* stamen filament growth after emasculation and hormonal application

When the floral buds of both *ms33* and WT plants were 1.5 - 2.0 mm long and the filament length was 0.5 - 0.8 mm (i.e., pollen maturation stage), the buds were gently opened using a fine forceps under a dissecting microscope, and the anthers were removed from stamens. The tops of filaments were covered with a lanolin (Fisher Scientific) cream made with water (control) or a plant hormone solution (1:1 w/w) using a fine glass needle under a dissecting microscope. Based on the volume of the cream applied, the approximate amount of hormone per filament was calculated as follows: 7 nmol GA_3, 14 nmol IAA, 11 nmol zeatin, 9 nmol ABA and 17 nmol ethrel. After 4 days of the treatment, the lengths of 30 filaments and the lengths of 200 epidermal cells (from 20 filaments) were measured.

II.7.2. *in vitro* culture of filaments with or without anthers

Floral buds of the same size as in II.7.1. were removed from *ms33* and WT plants and young stamens with 0.5 - 0.8 mm long filaments were excised from buds. Stamens were divided into two groups: 1. Intact stamens, and 2. Stamens in which anthers were removed. Both groups were cultured separately in 6 cm Petri dishes lined with two layers of filter paper containing 2 ml of Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) with or without one of the hormones (GA_3, IAA and zeatin). The concentration of hormones in the medium was 1.0 mg/l. The Petri dishes were sealed with Parafilm (American National Can, Neenah, WI, U.S.A.) and placed in a growth chamber in which conditions were set as in II.1. The lengths of filaments were measured after 72 h culture. Each treatment was repeated two times with 20 stamens or filaments.

II.8. *In vitro* plant growth

WT and *ms33* seeds were sterilized with 20% Javex for 10 min and rinsed 3 times with sterilized distilled water. The sterilized seeds were sown in plastic culture boxes (Magenta GA7 vessels, Sigma) containing 60 ml of the modified MS medium (the inorganic macroelements were reduced to 50%) and with both sugar and agar at 10 g/l each. The pH of the medium was adjusted to 5.8 with 1 N NaOH and the culture boxes containing the medium were autoclaved at 121°C for 20 min. The concentration of GA_3 or GA_4 in the medium was 1.5×10^{-5} M. The boxes containing seeds (5 seeds/box) were placed in a growth chamber with the same conditions as II.1. Sixty

seeds were used for each treatment, and plant growth was examined after 25 days of culture.

II.9. Seed germination

The seeds of WT and pure line of *ms33* mutant (obtained from low temperature treatment) were germinated in 6 cm (diameter) Petri dishes lined with two layers of filter paper (VWR Scientific Products). Fifty seeds were germinated in each dish containing 2 ml of distilled water, or a plant hormone solution containing 0.02% (v/v) Tween-20. Seeds were exposed to different light and temperature conditions, and plant hormones and/or a growth regulating substance (see below) at 24°C for 7 days. The germination rate was recorded every day for 7 days. For the dark treatment, the germination was examined under a green light. Each experiment was repeated 3 times.

II.9.1. Light and temperature treatments

WT and *ms33* seeds were distributed on filter paper soaked with water in Petri dishes. The Petri dishes were placed either in the dark, in white light (fluorescent tubes, Osram Sylvania Ltd., Versailles, KY, U.S.A.), in red light [fluorescent tubes wrapped with red film (Ruby lithography, Transalwrap Inc. Toronto, Canada) which transmits spectrum from 600 nm to 800 nm] or in blue light [tungsten bulb (Philips, Canada Ext. Services) wrapped with a blue film with transmission peak at 450 nm]. The light intensities of white, red and blue light were 120, 10 and 8 μmol m^{-2} s^{-1}, respectively. Illuminating period for all light treatments was 16 h/day. For temperature treatments,

petri dishes were exposed to either 4°C or 15°C in the dark for 3 days, and then transferred to 24°C in dark for germination.

II.9.2. Hormone and growth regulator treatments

WT and *ms33* seeds were germinated in the presence of 10^{-3} and 10^{-4} M GA_3 or GA_4 solution in the dark. In another experiment, different concentrations of paclobutrazol (also called PP333), an inhibitor of GA biosynthesis (for source see Table 2), and a mixture of 10^{-4} M GA_4 and 10^{-4} M paclobutrazol solution, were used.

II. 10. Analyses of endogenous plant hormones

II.10.1. Sample collection

The mature flowers of *ms33* mutant and WT plants grown at normal temperature (as described in II.1) and low temperature (15/11°C) were collected separately. During the collection, flowers were placed in 20 ml precooled plastic vials on ice. When the vials were full, they were stored in a -80°C freezer immediately. The vials for each sample were combined and ground into a fine powder with a Polytron homogenizer [Kinematica PT 10/35, Brinkmann Instruments (Canada) Ltd.] in a beaker containing liquid nitrogen. The ground samples were freeze-dried in a freeze dry system (VirTis, Gardiner, New York). The dried samples were stored in a -80°C freezer for subsequent analysis.

II.10.2. GA analysis

II.10.2.1. Extraction of GAs

One gram of dry sample was soaked in a mortar containing 20 ml of 80% aqueous methanol (MeOH), and internal standards of GAs, i.e., $[^2H_2]$-GA_1 10 ng, $[^2H_2]$-GA_3 10 ng, $[^2H_2]$-GA_4 20 ng, $[^2H_2]$-GA_9 20 ng, $[^2H_2]$-GA_{15} 20 ng and $[^2H_2]$-GA_{20} 20 ng, were added at this time. The sample was ground in a mortar with a pestle for 10 min at room temperature and the extracted solution was collected through a filter funnel. The residue was re-extracted two times with the same volume of 80% MeOH. All filtrates were combined and dried under reduced pressure on a rotary evaporator (Rotavapor RE 111, Büchi Laboratoriums-Technik AG, Switzerland) at 35°C. The dried residue was dissolved in 1 ml of 100% MeOH.

II.10.2.2. Purification of GAs

A 15 ml column was filled with 8 g of Prep. C_{18} (125 Å, 55-105 μm), and washed with 50 ml of 100% MeOH and 50 ml of 80% MeOH, respectively. The above sample solution was loaded on the column and 20 ml of 80% MeOH was used to elute the column. The eluate was collected and dried as above. The residue was dissolved in 1 ml of 100% MeOH and mixed with 1 g Celite-545 in a beaker. The mixture was dried with warm air and the dried sample loaded on a 20 ml column filled with 5 g of ICN Silica (100 mesh) and suspended in ethyl acetate (EtOAc) and hexane (95:5 v/v). The column was eluted with 80 ml of EtOAc:hexane (95:5 v/v) and the eluate was dried at the same conditions as above. The residue was dissolved in 1 ml of 100% MeOH.

In the dissolved sample, a 0.5 ml solution of 1% acetic acid (HAc) was added and ^{3}H-labeled GAs {[^{3}H]-GA_1 ~50,000 DPM, [^{3}H]-GA_4 ~50,000 DPM and [^{3}H]-GA_9 ~50,000 DPM} were added as radiotracers. The sample solution was filtrated into a 2 ml glass vial by a syringe filter (Waters, U.S.A.). The residue was washed with 0.5 ml of 100% MeOH and the solution was subjected to high performance liquid chromatography (HPLC) using the Waters M-45 system consisting of 2 Waters 510 pumps, a U6K injector and a photodiode array detector connected to a computer. Various fractions were collected on a Gilson 201 fraction collector.

(i) Conditions for reverse-phase HPLC: Reverse-phase HPLC was performed using the C_{18} RCM column (8 x 110 mm, 5 μ, Whatman Partisphere, Clifton, NJ, U.S.A.). The column was washed with 100% MeOH and equilibrated with 10% MeOH by a linear gradient. After the sample was injected, the column was eluted isocratically with a solvent mixture of 10% MeOH and 100% MeOH (40:60) for 40 min at a flow rate of 1 ml/min. The eluate was collected in 1 ml/fraction and 10 μl solution of each fraction was mixed with 5 ml of scintillant to detect radioactivity using a Scintillation Analyzer (Tri-Carb 2200 CA, IL, U.S.A.). The fractions were grouped based on the peaks of radioactivity. All groups were dried separately and each residue was dissolved in 1 ml of MeOH : HAc (99.9 : 0.1 v/v). The samples were further purified with a normal-phase HPLC.

(ii) Conditions for normal-phase HPLC: A normal-phase column [Nucleosil $N(CH_3)_2$, 5 μ, 150 x 4.6 mm, Alltech, U.S.A.] was used. The column was washed with 100% MeOH and equilibrated with MeOH : HAc (99.9 : 0.1 v/v). After the sample was

injected, the column was eluted with MeOH : HAc (99.9 : 0.1 v/v) for 40 min at a flow rate of 1 ml/min. Every 1 ml of eluate was collected as a fraction and the radioactivity in each fraction was detected as above. The fractions were combined according to the peaks of radioactivity and dried.

II.10.2.3. Methylation of GAs

To the dried residue containing GA_9 and GA_{15}, 20 drops of diazomethane (in ether) were added. The reaction was allowed at room temperature for 30 min in a fume hood, and then, the mixture was dried with nitrogen gas. For the samples containing GA_1, GA_3, GA_4 and GA_{20}, diazomethane was first used as a methylation reagent to methylate carboxylic acid groups of GAs, and after drying, 3 drops of pyridine and 6 drops of bis-trimethyl-silyltrifluoroacetamide were added to methylate hydroxyl groups of GAs. Air in the reaction vial was removed with nitrogen gas. The reaction vial was heated at 75°C for 40 min, and the sample was dried with nitrogen gas.

II.10.2.4. Quantitative analysis of GAs

Each GA was quantified by gas chromatography-mass spectrometry-selected ion monitoring (GC-MS-SIM). The dried sample was dissolved in 2 drops of hexane. 2 μl of the dissolved sample was introduced into a GC-MS (GC, Hewlett Packard 5890 II; MS, Hewlett Packard 5970 A) by on-column injection into a retention gap of 0.5 m x 0.32 mm deactivated fused silica capillary DB1-15N column (15 m x 0.25 mm, 0.25 μm methyl silicone film, J. & W. Scientific, Folsom, CA, U.S.A.). The oven was heated

ballistically from 60°C to 200°C at 20°C/min and from 200°C to 280°C at 5°C/min. Data acquisition was controlled by a HP 300 Series computer. The levels of GAs were calculated by the ratio of the characteristic ions of endogenous GAs and internal standards of GAs, respectively. The characteristic ions of GAs/ $[^2H_2]$-GAs were selected as follows: GA_1, M^+ 506/508; GA_3, M^+ 504/506; GA_4, M^+ 418/420; GA_9, M^+ 298/300; GA_{15}, M^+ 344/346; GA_{20}, M^+ 418/420.

II.10.3. Analyses of IAA and ABA

II.10.3.1. Extraction and purification by reverse-phase column chromatography

The procedures for extraction and purification by a reverse-phase C_{18} open column for IAA and ABA were the same as that for GAs, but 200 ng $[^{13}C_6]$-IAA and 100 ng $[^2H_6]$-ABA were added as internal standards.

II.10.3.2. Purification by partitioning

The dry sample from the last step was dissolved in 10 ml of 1% HAc. The acidic solution was partitioned with 10 ml of EtOAc (saturated with 2% HAc) three times. The EtOAc solutions were combined and dried. The residue was dissolved in 1 ml of 100% MeOH and 1 ml of 1% HAc. $[^3H]$-IAA (50,000 DPM) and $[^3H]$-ABA (50,000 DPM) were added as radiotracers for collection of fractions.

II.10.3.3. Purification by Sep-pak C_{18} column

Sep-pak C_{18} Cartridges (Waters Associates, Milford, MA, U.S.A.) were washed with 100% MeOH and 50% MeOH, respectively. The sample solution above was loaded on the column with a syringe. The column was eluted with 15 ml of 50% MeOH, and the eluate collected was dried.

II.10.3.4. Purification by HPLC

The dry sample was dissolved in 0.1 ml of 100% MeOH, and then mixed with 0.9 ml of 1% HAc. The mixture was filtered with a syringe filter. The sample solution was injected into HPLC with a C_{18} RCM column (8 x 110 mm). The solvent system for elution was 10% MeOH in Pump A and 100% MeOH in pump B and the flow rate was 2 ml/min. The following elution program was set: From 0-10 min, 100% of pump A; from 10 to 40 min, 30% of pump A and 70% of pump B with a linear gradient; from 40 to 50 min, 0% pump A and 100% pump B. The fractions were collected as 10 ml/tube in the first 4 tubes, followed by 3 tubes of 5 ml/tube, 5 tubes of 2 ml/tube and 7 tubes of 5 ml/tube. The radioactivity in each tube was detected, and the tubes were combined and dried separately. IAA and ABA samples were methylated with diazomethane by the same procedure as that for GA_9 and GA_{15}.

II.10.3.5. Quantitative analysis

IAA and ABA were quantified by GC-MS-SIM. The equipment used was the same as that for GAs (II.10.2.4). The temperature was programmed from 15°C to 195°C

at 15°C/min, and from 195°C to 275°C at 5°C/min. For quantification of IAA and ABA, calculation was based on the ratios of characteristic ions of endogenous IAA, ABA and their internal standards. The following characteristic ions were recorded: IAA, M^- 189/195; ABA, M^- 190/194.

II.10.4. Sources of chemicals used in hormone analyses

The sources of chemicals used in the hormone analyses are listed in Table 3.

II.11. Construction of double mutants

Plants homozygous recessive for *ms33* were crossed with mutant plants homozygous recessive for *aba-1* and *spy-3* to generate *ms33/ms33 aba-1/aba-1*, and *ms33/ms33 spy-3/spy-3* double mutants. The F_2 seeds were collected and sown in pots. Novel phenotypes with characteristics of both the parent mutants were identified from the F_2 population. Other phenotypes in the F_2 population were also scored. Chi-square analysis was used to determine the significance of the dihybrid ratio (9:3:3:1).

II.12. Statistical methods

The data on filament and cell lengths for all experiments were analyzed by the analysis of variance. In other cases, standard errors of the means were calculated.

Table 3. Sources of chemicals used in hormone analyses

Chemical	Source
bis-Trimethyl-silyltrifluoroacetamide	Sigma Chemical
Celite-545	"
$[^2H_6]$-ABA	Dr. M. Saugy, Switzerland
$[^3H]$-ABA, $[^3H]$-IAA, $[^3H]$-GA_1, $[^3H]$-GA_4	Amersham Pharmacia Biotech
$[^3H]$-GA_9	Dr. Alan Crozier, U.K.
$[^{13}C_6]$-IAA	Dr. J. Cohen, USDA
$[^2H_2]$-GA_1, $[^2H_2]$-GA_3, $[^2H_2]$-GA_4, $[^2H_2]$-GA_9, $[^2H_2]$-GA_{15}, $[^2H_2]$-GA_{20}	Dr. L. Mander, Australia
ICN Silica	Sigma Chemical
Prep. C18	Waters, Milford

III. RESULTS

III.1. Morphology of WT and *ms33* flowers

As reported previously (Clark and Meyerowitz, 1994), WT *Arabidopsis* flowers consist of 16 floral organs in four whorls. In the outermost whorl 1, four green sepals develop. Inner to and alternating with the sepals are four white petals in whorl 2. Whorl 3 consists of six stamens; two pairs of long medial and two short lateral stamens. The innermost whorl 4 has two fused carpels that form the gynoecium (Fig. 1A).

In *ms33* flowers, all floral organs were present as in WT and in the same order. The striking difference was that the stamens were short in length and anthers contained few pollen grains (Fig. 1B). Before the mutant floral buds opened (Fig. 1C), the sepals and gynoecium were longer than, but petal and stamen lengths were similar to, those in WT buds (Table 4). In WT flowers immediately before anthesis, there was rapid growth of petals and stamens, and at anthesis, the long stamens extended beyond the level of the stigma (Fig. 1A). However, in the mutant buds, petal elongation was delayed by approximately 7 days, but the gynoecium elongated and protruded through the bud before anthesis (Fig. 1D). Further, the growth of stamens was inhibited in mutant flowers and at maturity the stamens barely reached the mid-position of the gynoecium (Fig. 1B). A comparison of floral organs at anthesis showed no difference in petal lengths between *ms33* and WT flowers (Table 4). However, the mutant stamens were significantly shorter, sepals and gynoecium longer, than the respective WT organs

Fig. 1. WT and *ms33* flowers. A: Mature WT flower with 4 sepals, 4 petals and 4 long stamens that were beyond the stigma level, and 2 short stamens. Each anther had plenty of pollen. The gynoecium was in the centre of the flower. (x 40). B: Mature *ms33* flower showing short stamens with some pollen (x 36). C: *ms33* floral bud just before opening (x 16). D: *ms33* floral bud showing gynoecium growth before anthesis (x 14).


A
B
C
D

Table 4. The lengths (cm) of floral organs in *ms33* and WT buds before opening, and in mature flowers at anthesis. Each value is a mean ± SE. n = 20 for each organ type.

	Organ type	WT	*ms33*
Unopened floral bud	Sepal	2.01 ± 0.02	2.14 ± 0.02*
	Petal	1.81 ± 0.03	1.75 ± 0.02
	Long median stamen	1.36 ± 0.02	1.39 ± 0.02
	Gynoecium	1.89 ± 0.02	2.02 ± 0.02*
Mature Flower	Sepal	2.04 ± 0.03	2.18 ± 0.02*
	Petal	3.39 ± 0.04	3.35 ± 0.04
	Long median stamen	2.87 ± 0.02	1.72 ± 0.02*
	Gynoecium	2.44 ± 0.05	3.32 ± 0.06*

* indicates significantly different from the WT at $P < 0.01$.

(Table 4, Fig. 2A and C). There was a corresponding reduction in epidermal cell length of mutant filaments compared to WT (Fig. 2B and D).

III.2. Pollen morphology and germination of WT and *ms33*

WT flowers produced a large amount of pollen (1028 ±31 grains/anther) which were released at anthesis. Mature pollen grains generally had three furrows (Fig. 3A). The anthers of *ms33* produced relatively small amount of pollen (592 ± 27 grains/anther) at anthesis, and pollen dehiscence was delayed. *ms33* pollen were also non-viable as they failed to induce fruit- and seed-set by manual pollination. The *ms33* pollen grains were approximately 20% longer than that of WT pollen. There were surface markings apparent on mutant pollen wall, but the furrows were not clear (Fig. 3B).

The total of 7,546 pollen grains collected from 10 WT flowers showed, on average, 65% germination in the Hodgkin and Lyon's (1986) medium. In contrast, none of the 6,382 pollen grains sampled from 10 mutant flowers germinated in the same medium.

III.3. Growth of WT and *ms33* plants

WT seeds sown under normal growth conditions (22/18°C and 16/8 h photoperiod, day/night) started to germinate after 2 days. After 3 weeks of vegetative growth, when the ninth leaf had emerged, plants began to bolt and entered the reproductive phase. Each plant produced a primary inflorescence and 4-5 secondary

Fig. 2. SEM of WT and *ms33* long stamens and filaments. A: A WT long stamen (x 31); B: Mid-portion of a WT stamen filament (x 420); C: *ms33* long stamen (x 32); D: Mid-portion of an *ms33* stamen filament (x 430). B and D show the epidermal cell profile of WT and *ms33* filaments.

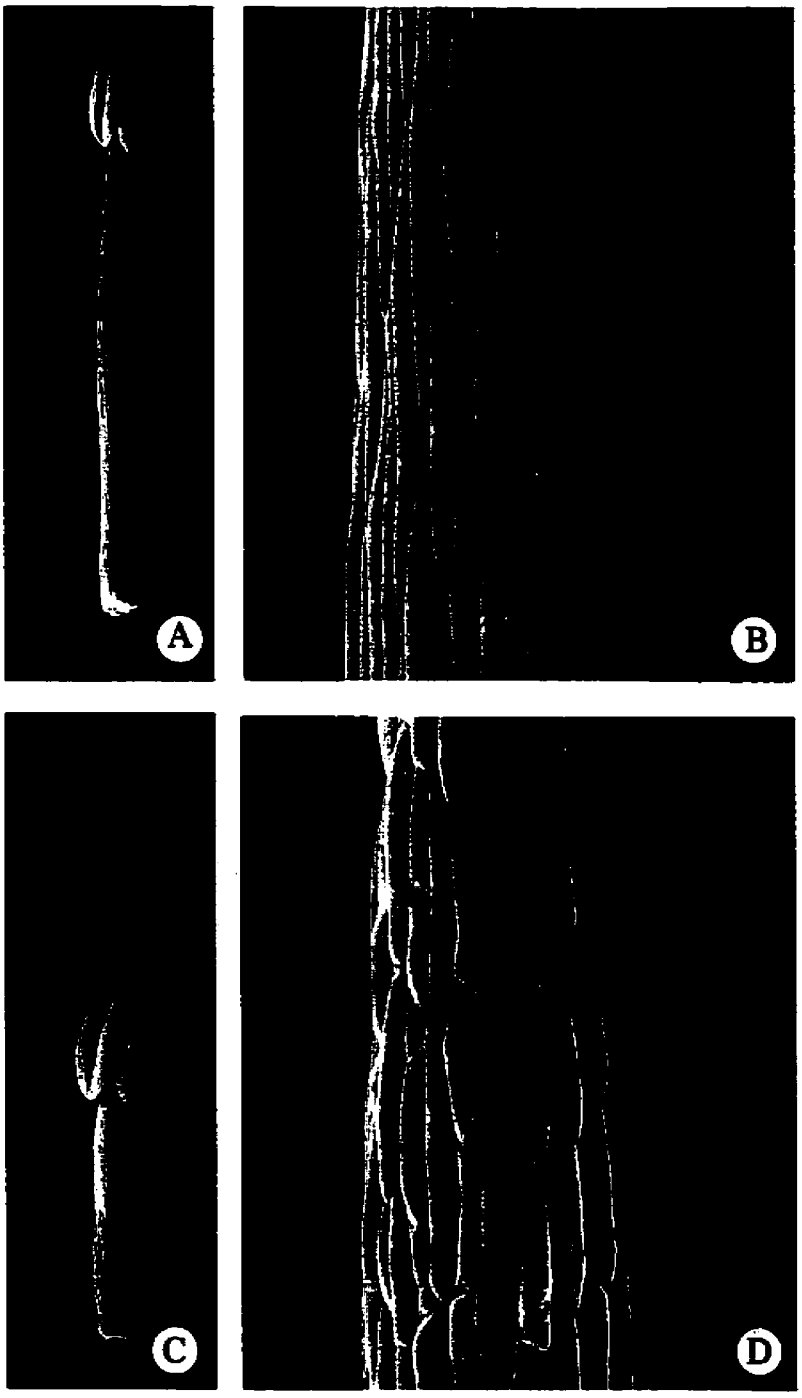
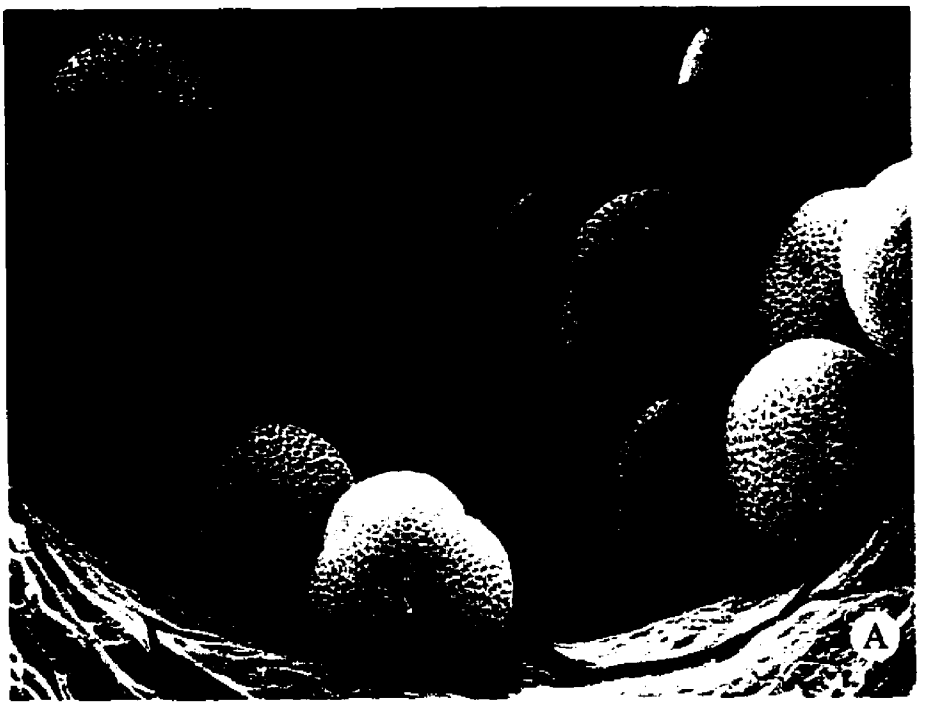
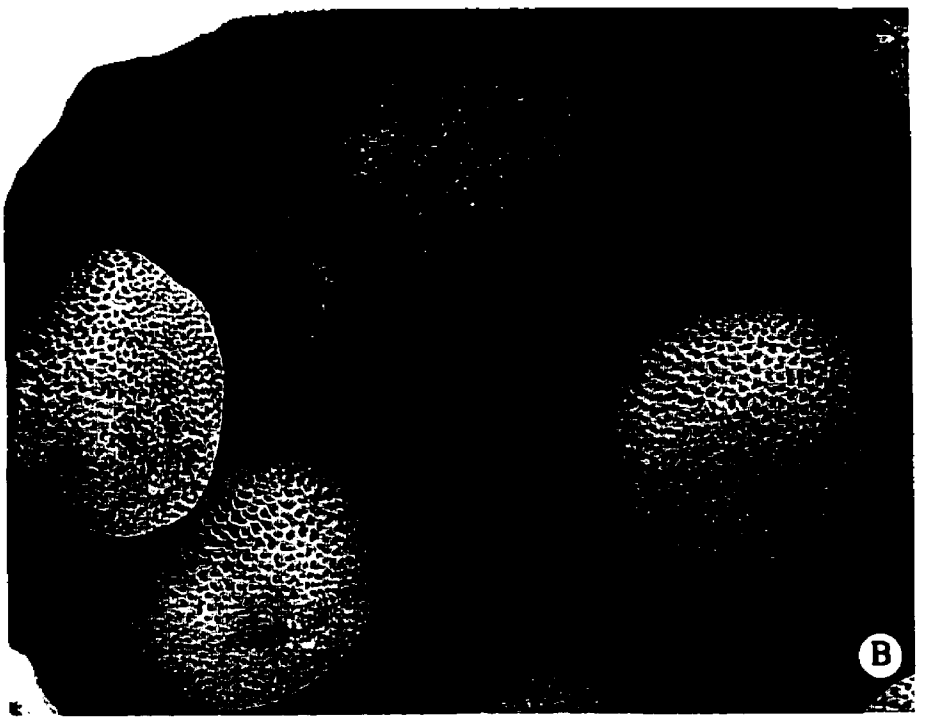

A
B
C
D

Fig. 3. SEM micrographs of WT and *ms33* pollen grains. A: WT pollen grains showing three furrows on each grain (x 2,500). B: *ms33* pollen grains which are larger in size than WT show surface markings, but furrows are not clear (x 2,500).


A


B

inflorescences. A number of siliques developed subsequently, each with different number of seeds (max. 72). The major part of plant height was contributed by the peduncle and it reached approximately 23 cm at the end of 7 weeks of growth (Fig. 4). In WT plants, the time from first flower opening to seed maturation was approximately 4 weeks. Thus, the life span of WT plants was around 7 weeks under our growth conditions.

Pure line *ms33* mutant seeds (obtained from low temperature treatment, see section III.5.5.2) were sown in pots and grown in the same growth conditions as the WT plants. There was an apparent delay in germination of *ms33* seeds (for details on the kinetics of seed germination, see section III.5.6) which led to slow vegetative growth and late flowering (by approximately 5 days), as compared to WT plants (Fig. 4, Fig. 5A, B and C). However, if *ms33* seeds were exposed to 4°C for 3 days before germination, the vegetative growth and flowering time of *ms33* plants were similar to WT plants (Fig. 5D, E and F). There were no gross phenotypic differences in the vegetative organs between *ms33* and WT plants developed from seeds exposed, or not exposed, to 4°C for 3 days before germination (Fig. 5B and E).

III.4. Tapetum and pollen development in *ms33* and WT

As indicated above, *ms33* mutant produces a small amount of pollen (Fig. 1B), and the pollen is both abnormal in shape (Fig. 3B) and is non-viable. Dawson et al. (1993) examined pollen development in *ms33* at the light microscope level and found that the breakdown in pollen development occurs during the final maturation stage. To

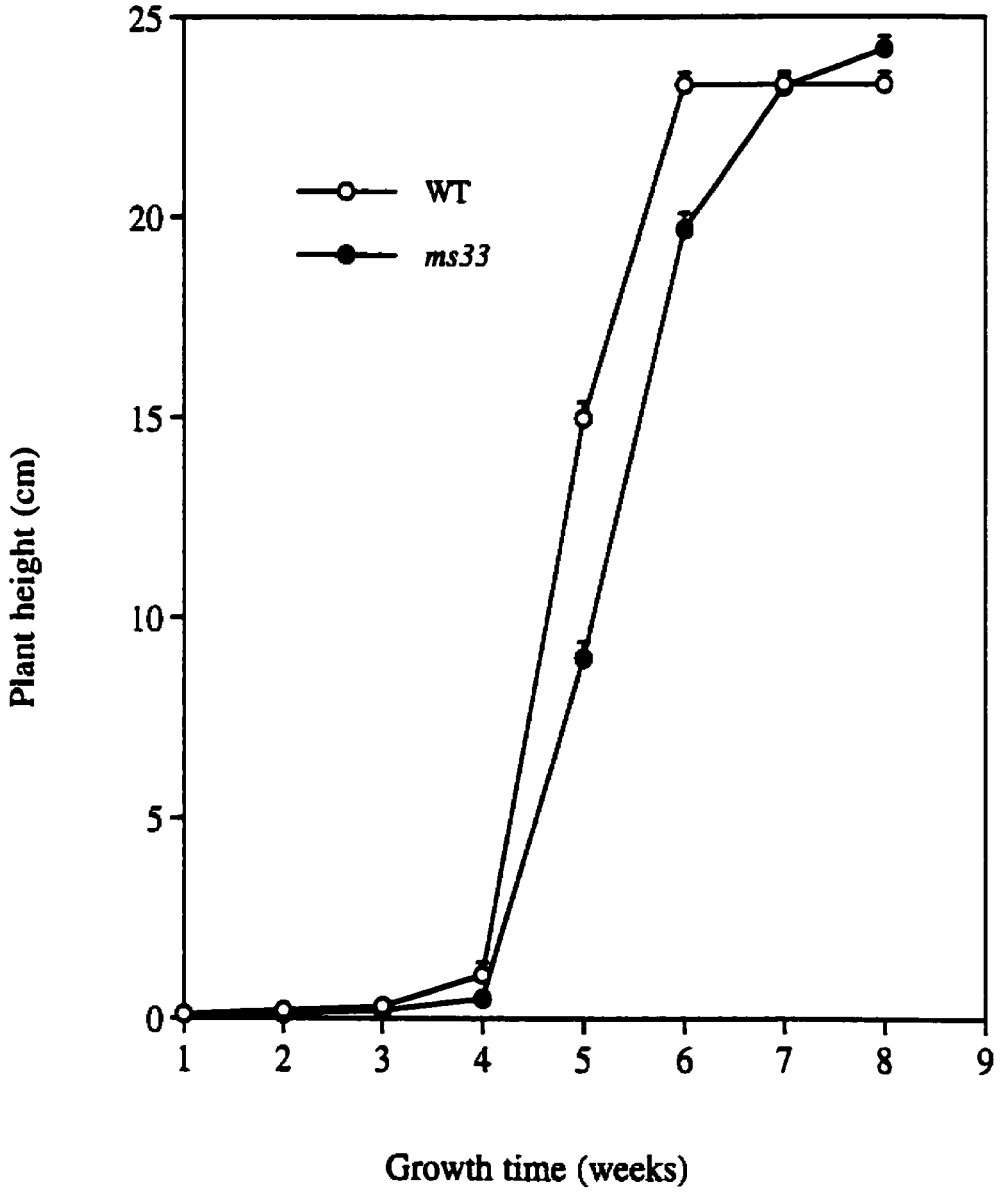
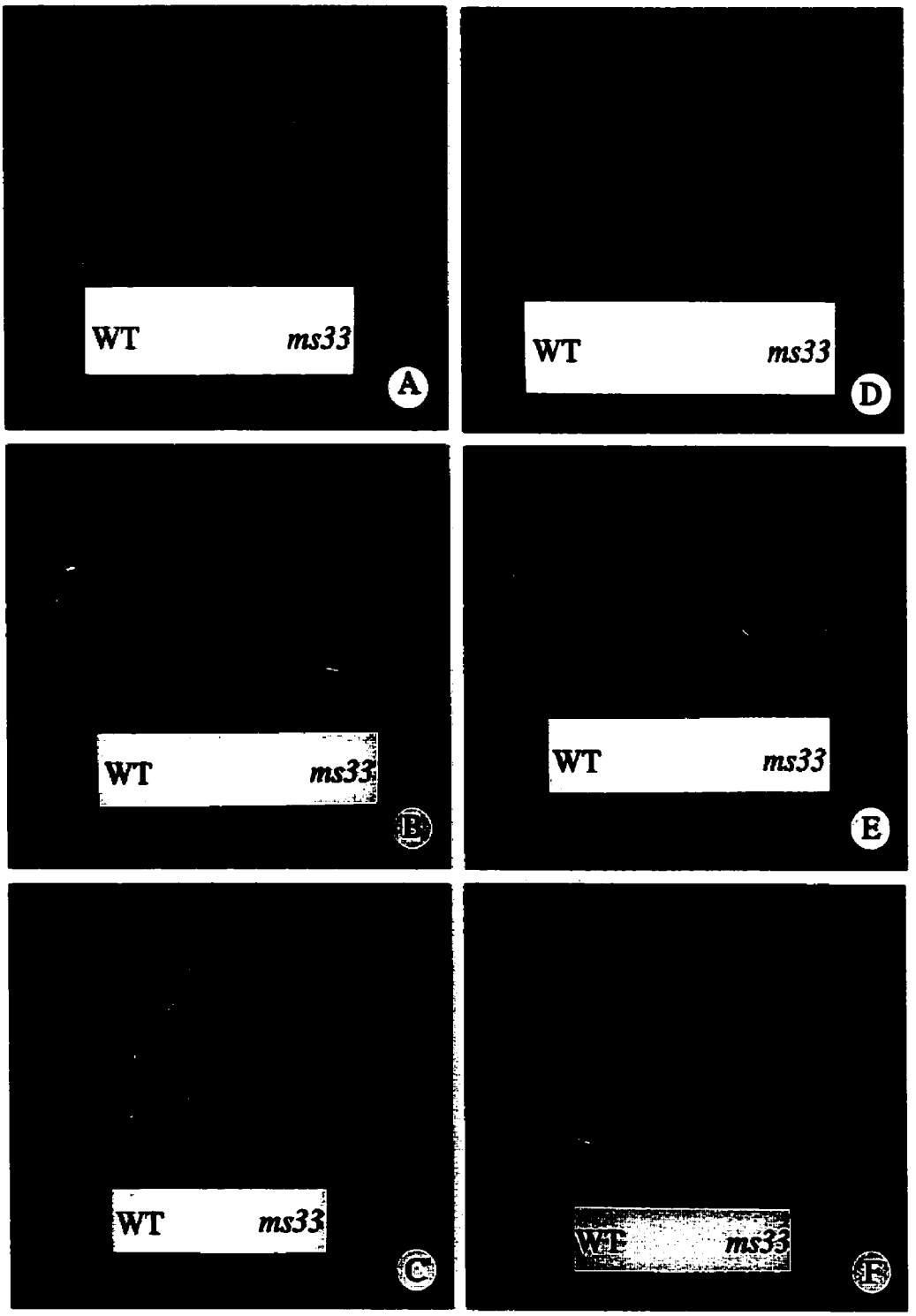


Fig. 4. The height of WT and *ms33* plants sown and grown at 22/18°C and 16/8 h photoperiod (d/n). Each value is a mean of 30 plants. Bars indicate S.E.

Fig. 5. WT and *ms33* plants grown at 22/18°C and 16/8 h photoperiod (d/n). Seeds of both genotypes were either exposed, or not exposed, to 4°C for 3 days before germination. A, B and C: WT and *ms33* plants grown from seeds not exposed to 4°C for 3 days before germination. In 5C, *ms33* mutant plants show late flowering. D, E and F: WT and *ms33* plants from seeds exposed to 4°C for 3 days before germination show no difference in the growth and flowering time. A and D = 2 weeks, B and E = 4 weeks, C and F = 5 weeks of growth.


WT
ms33
A
WT
ms33
D
WT
ms33
B
WT
ms33
E
WT
ms33
C
WT
ms33
F

further understand the nature of male sterility in this mutant, a comparative ultrastructural study of the tapetum and pollen development in *ms33* and WT anthers was conducted. Although pollen development is a continuous process, for ease of description, it is usually divided into a number of stages, from sporogenous cell stage to mature pollen. For example, six stages in *Brassica napus* (Polowick, 1989), eight in soybean , nine in maize (Palmer et al., 1992), and twelve in *Arabidopsis* (Owen and Makaroff, 1995) were divided. Since the pollen abortion in *ms33* occurs during pollen maturation, eight stages, i.e., four from sporogenous cell to microspore stages, and four during pollen maturation, were examined in the present work.

III.4.1. Tapetum and pollen development in WT

Although WT pollen development, both at LM and TEM levels, in different ecotypes of *Arabidopsis* has been reported earlier (Regan and Moffatt, 1990; Dawson et al., 1993; Owen and Makaroff, 1995; Zajac, 1997; Taylor et al., 1998), a comparative study of tapetum and pollen development in the *ms33* mutant and WT of the same ecotype (Landsberg *erecta*) and grown in our conditions, was conducted.

III.4.1.1. Sporogenous cell stage

There were four layers of cells surrounding the sporogenous tissue in a cross section of the anther, i.e., epidermis, endothecium, middle layer and tapetum, from the outside to inside of the anther (Fig. 6A). The tapetal cells contained numerous plastids, mitochondria and rough endoplasmic reticulum (RER), and the nucleus had a large

Fig. 6. Sporogenous cell stage in WT anther. A: LM of a cross section of a WT anther (x1,010). B: TEM micrograph of tapetal cells (x 11,800). C: TEM micrograph of a sporogenous cell (x 9,400).

En = endothecium, Ep = epidermis, M = mitochondria, Mi = middle layer, N = nucleus, Nu = nucleolus, P = plastid, R = RER, S = sporogenous tissue, T = tapetum, V = vacuole.

A
V
B
C

nucleolus (Fig. 6B). Tapetal cells also possessed one or more vacuoles (Fig. 6A and B). The sporogenous cells were rich in mitochondria, had a few large plastids, long strands of RER and small vacuoles (Fig. 6C). A distinct nucleolus was present in the nucleus.

III.4.1.2. Pollen mother cell (PMC) stage

The cytoplasm of WT tapetal cells was more dense at the PMC stage than at the earlier stage, and contained many mitochondria, plastids, RER, and both a large vacuole and some small vacuoles (Fig. 7A and B). In the PMCs, a large nucleus with a nucleolus occupied the central area of the cell (Fig. 7C). Numerous mitochondria, plastids and small vacuoles were dispersed throughout the cytoplasm, but RER was reduced in amount as compared to the previous stage. There was a layer of callose deposited closer to the primary cellulose wall of PMCs, and an electron-lucent space was observed between the callose wall and plasmalemma (Fig. 7A and C). This space is likely not a fixation artifact since it has been observed in other studies on *Arabidopsis* microsporogenesis (Chaudhury et al., 1994; Peirson et al., 1996).

III.4.1.3. Tetrad stage

At the tetrad stage, cytoplasm of tapetal cells was still dense and contained large vacuoles (Fig. 8A). The major features of this stage were; 1. The tapetal cells were binucleate, and 2. they contained stacked RER (Fig. 8B and C) which surrounded the nuclei. The number of RER per stack ranged from 2 to 8 (Fig. 8B). Other organelles, e.g. plastids and mitochondria, were of normal structure and distribution in tapetal cells.

Fig. 7. Pollen mother cell stage in WT anther. A: LM of a cross section of a WT anther (x 1,010). B: TEM micrograph of a tapetal cell (x 9,200). C: TEM micrograph of a pollen mother cell (x 9,050).

C = callose, M = mitochondria, N = nucleus, Nu = nucleolus, P = plastid, R = RER, V = vacuole.

A
V
R
N
Nu
M
B
P
M
C

Fig. 8. Tetrad stage in WT anther. A: LM of cross section of a WT anther (x 1,030). B: Stacked rough ER in a tapetal cell (x 51,600). C: TEM micrograph of a WT tapetal cell (x16,000). D: TEM micrograph of a tetrad (x 7,800).

C = callose, CW = cellulose wall, Ex = exine, M = mitochondria, N = nucleus, Nu = nucleolus, P = plastid, R = RER, SR = stacked RER, V = vacuole.

A
M
SR
P
C
D
D

Microspores in the tetrad were separated by a thick callose wall and the electron-lucent space between the callose wall and plasmalemma was reduced as the callose wall gradually filled that space (Fig. 8D, see also Peirson et al., 1996). A primary cellulose wall surrounding the tetrad was still visible. The cytoplasm of each microspore was rich in mitochondria, plastids, small vacuoles and RER. The early signs of exine formation were noticeable at this stage.

III.4.1.4. Microspore stage

The tapetal cells still contained a large vacuole, were binucleate, and had a normal complement of organelles (Fig. 9A and B). Stacked RER around the two nuclei were still evident, but there were fewer RER per stack than at the tetrad stage (Fig. 9B). In the microspores released from tetrads, the distribution of organelles, i.e., mitochondria, plastids, small vacuoles, and RER was the same as the earlier stage, and there was a large nucleus with a nucleolus (Fig. 9C). In the exine, the foot layer had been formed, but tecta and columellae were not completely developed. The osmiophilic deposits, presumably released from the tapetum, were visible in the anther locule and deposited on the outside of exine.

III.4.1.5. Vacuolate microspore stage

The cytoplasm of tapetal cells was dense and there were many vacuoles in the tapetal cells at this stage (Fig. 10A and B). The nuclei were intact and mitochondria were normal. However, the RER was further reduced as compared to that at the tetrad

Fig. 9. Microspore stage in WT anther. A: LM of a cross section of a WT anther (x 1,030). B: TEM micrograph of a tapetal cell (x 10,300). C: TEM micrograph of an early microspore (x 14,100).

Ex = exine, M = mitochondria, N = nucleus, Nu = nucleolus, OD = osmiophilic deposits, P = plastid, R = RER, SR = stacked RER, V = vacuole.

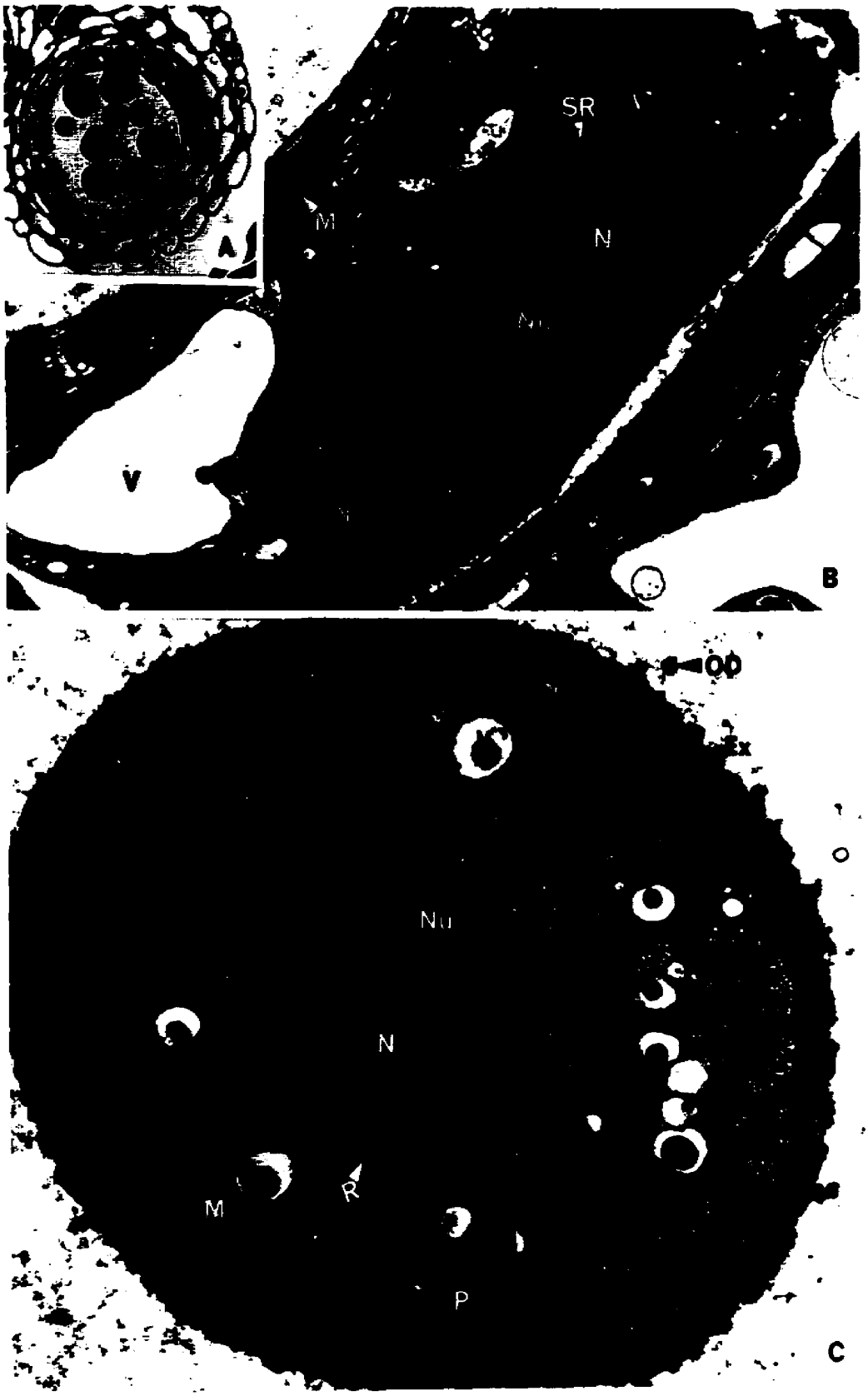

SR
M
N
V
B
Nu
N
M
R
P
C

Fig. 10. Vacuolate microspore stage in WT anther. A: LM of a cross section of a WT anther (x 1,110). B: TEM micrograph of a tapetal cell (x 14,000). C: TEM micrograph of a vacuolate microspore (x 10,300).

Ex = exine, M = mitochondria, N = nucleus, Nu = nucleolus, P = plastid, R = RER, SR = stacked RER, V = vacuole.

V
P
N
M
Nu
SR
N
A
B
P
Nu
V
Ex
C

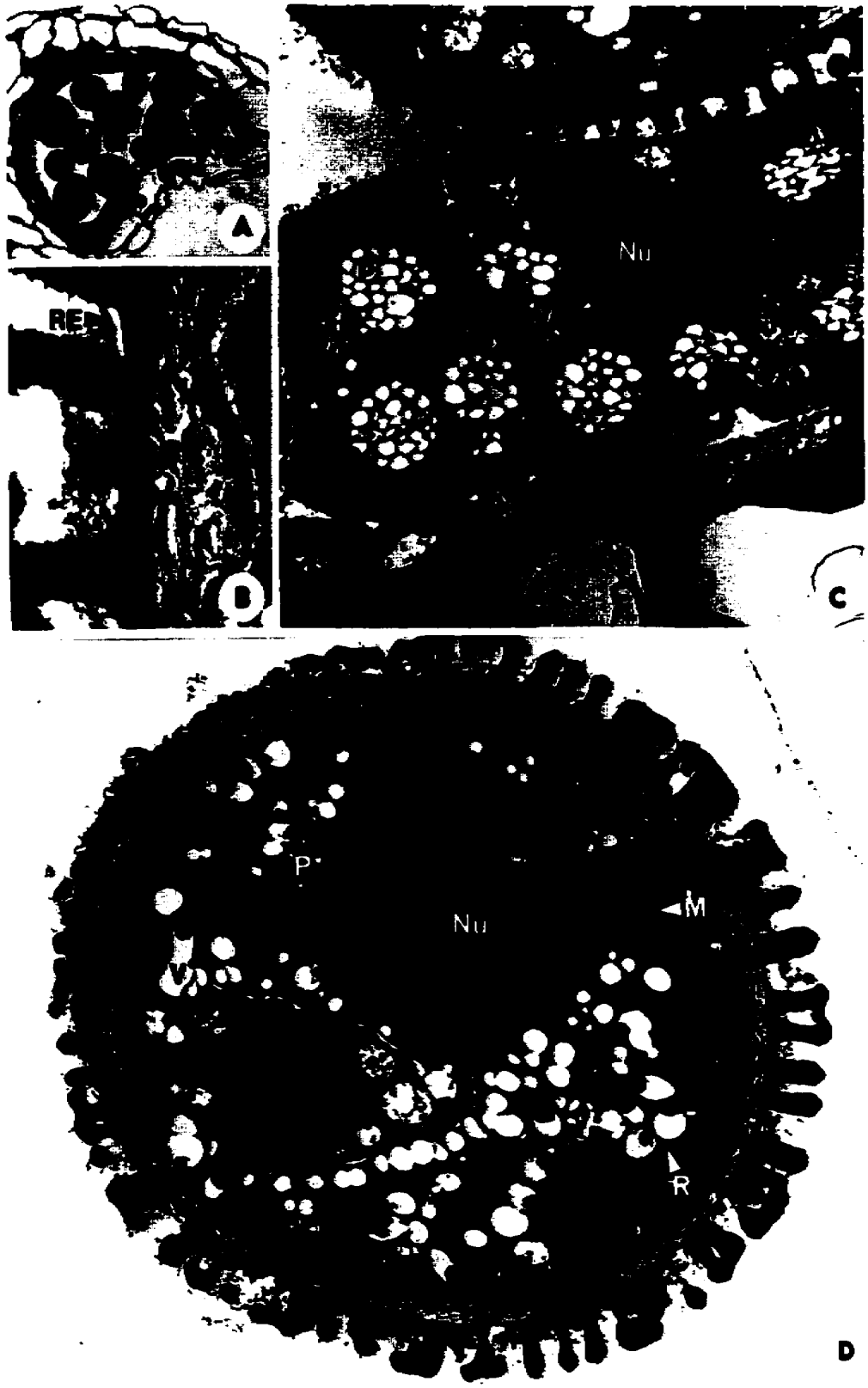
and microspore stages (Fig. 8B, 9B). Plastids had become swollen and contained numerous small electron-transparent globuli (Fig. 10B). The microspores normally contained a large vacuole which displaced the nucleus to one side of the cell (Fig. 10C). The nucleus had a nucleolus and the cytoplasm of microspores contained mitochondria, plastids and RER. The exine was well developed at this stage and had columellae and tecta.

III.4.1.6. Bicellular pollen stage

At this stage there were no large vacuoles in the tapetal cells, and the structure of mitochondria and the nucleus was still normal (Fig. 11A and C). A distinctive cytological change in the tapetal cells was the degeneration of plastids which possessed numerous large electron-transparent globular inclusions (Fig. 11C). Some vesicles with osmiophilic inclusions (also reported by Webb, 1992, and Owen and Makaroff, 1995) were also observed in the tapetal cells. In the bicellular pollen grains, the intine had two distinct layers. The exintine layer had substructures and the endintine was a homogeneous layer that appeared wavy (Fig. 11B). There were some rectangular electron-lucent and osmiophilic deposits between the columellae of the exine (Fig. 11D). The vegetative cell contained many mitochondria, small vacuoles and plastids, with some starch grains, and the nucleus contained a large and a small nucleolus (Fig. 11D). Within the bicellular pollen grain, the generative cell was detached from the intine, had a distinct wall, and occupied a central location in the vegetative cell. Some mitochondria and vacuoles were also observed in the generative cell, and it contained a

Fig. 11. Bicellular pollen stage in WT anther. A: LM of a cross section of a WT anther (x 370). B: TEM micrograph of a portion of pollen wall (x 36,000). C: TEM micrograph of a tapetal cell (x 8,000). D: TEM micrograph of a bicellular pollen grain (x 9,400).

Ei = exintine, En = endintine, Ex = exine, GN = generative nucleus, M = mitochondria, Nu = nucleolus, P = plastid, R = RER, RE = rectangular electron-lucent deposit, V = vacuole, Ve = vesicle, VN = vegetative nucleus.


A
RE
D
Nu
C
P
Nu
M
R
D

large nucleus with a nucleolus (Fig. 11D).

III.4.1.7. Tricellular pollen stage

At this stage, the tapetum became much reduced than the previous stages (Fig. 12A), and the cells showed clear signs of degeneration. The nucleus was not visible, and some plastids and vesicles were totally degenerated (Fig. 12C). However, the mitochondria were still intact. In the pollen grains, the thickness of exintine was reduced, but that of endintine had increased and was still wavy (Fig. 12B). The cytoplasm of the vegetative cell was more dense than at the earlier stage, but mitochondria and plastids (containing starch grains) were visible (Fig. 12D). The vacuoles were reduced in size and were more in number than at the bicellular pollen stage. Each of the two sperm cells was surrounded by a thin wall and contained dense cytoplasm.

III.4.1.8. Mature pollen stage

At the mature pollen stage the tapetal tissue had completely degenerated, and in the anther an opening at the area of septum and stomium had been formed (Fig. 13A). A large amount of tryphine (the term was used by Preuss et al., 1993) was deposited between the columellae and the outside of the pollen wall. In these deposits there were numerous electron-lucent structures (Fig. 13B). The endintine layer had become flat and the exintine had lost the visible substructures, present in the previous stages, but contained some osmiophilic material (Fig. 13B). In the vegetative cell, the vacuoles

Fig. 12. Tricellular pollen stage in WT anther. A: LM of a cross section of an WT anther (x 530). B: TEM micrograph of a portion of pollen wall (x 37,800). C: TEM micrograph of tapetal tissue (x 12,600). D: TEM micrograph of a tricellular pollen grain (x 7,600).

Ei = exintine, En = endintine, Ex = exine, M = mitochondria, Nu = nucleolus, P = plastid, R = RER, RE = rectangular electron-lucent deposit, SC = sperm cell, V = vacuole, Ve = vesicle, VN = vegetative nucleus,


A
B
RE
C
D
Nu
SC
SC
M

Fig. 13. Mature pollen stage in WT anther. A: LM of a cross section of an WT anther (x 710). B: TEM micrograph of a portion of the pollen wall (x 19,000). C: TEM micrograph of a mature pollen grain (x 5,000). D: TEM micrograph of a sperm cell (x 10,700). E: TEM micrograph of a portion of cytoplasm of vegetative cell of a mature pollen grain (x 16,300).

Ei = exintine, En = endintine, Ex = exine, L = lipid body, M= mitochondria, P = plastid, RE = rectangular electron-lucent deposit, SC = sperm cell, SG = starch grain, SM = sperm mitochondria, SN = sperm nucleus, Tr = tryphine, V = vacuole, VN = vegetative nucleus.

A
VM
SC
SC
C
Tr
D
D
E

were further reduced in size, and the nucleus was lobed (Fig. 13C). In the two sperm cells, nuclei and mitochondria were visible (Fig. 13D). The cytoplasm of the vegetative cell was rich in lipid bodies surrounded by a strand of RER, plastids containing starch grains, mitochondria and small vacuoles (Fig. 13E).

III.4.2. Tapetum and pollen development in the *ms33* mutant

Both the LM and TEM observations showed that the development of tapetum and microspores in *ms33* anthers was similar to the WT until the vacuolate microspore stage. The first cytological changes in *ms33* anthers were observed in the tapetal tissue at the bicellular pollen stage. Thus, the tapetum and pollen development in *ms33* anthers is described starting with the vacuolate microspore stage.

III.4.2.1. Vacuolate microspore stage

At the vacuolate microspore stage, the cytoplasm of tapetal cells in the *ms33* mutant was dense (Fig. 14A and B). Tapetal cells contained vacuoles, mitochondria, nuclei and swollen plastids in which there were many electron-transparent globuli (Fig. 14B). Microspores of *ms33* also contained a large vacuole, and the nucleus was displaced to one side of the microspore and contained a nucleolus (Fig. 14C). In the cytoplasm a number of mitochondria, plastids and RER were observed. The exine was well developed and the osmiophilic deposits were visible between columellae. These structural features of both the tapetum and microspores resembled those of the WT at the same stage.

Fig. 14. Vacuolate microspore stage in *ms33* anther. A: LM of a cross section of an *ms33* anther (x 760). B: TEM micrograph of a tapetal cell (x 15,000). C: TEM micrograph of a vacuolate microspore (x 11,200).

Ex = exine, M = mitochondria, P = plastid, N = nucleus, Nu = nucleolus, R = RER, SR = stacked RER, V = vacuole.

A
SR
P
Nu
V
B
C

III.4.2.2. Bicellular pollen stage

At the bicellular pollen stage, the tapetum showed signs of degeneration (Fig. 15A). The nucleus was absent, mitochondria and plastids had started to degenerate, and the vacuoles, present at the vacuolate microspore stage, had lost their identity (Fig. 15C). Thus, the degeneration of the tapetum in *ms33* was earlier than in the WT anther where the first signs of degeneration were observed at the tricellular pollen stage (Fig. 12C). A large amount of osmiophilic material was observed in the anther locule, presumably released from the degenerating tapetal tissue. There were also some distinct changes in *ms33* pollen grains at this stage. First, the exintine of the intine was much thinner than that in WT pollen and contained some osmiophilic structures (Fig. 15B). The endintine was relatively thick and wavy. Second, there were many vacuoles in the pollen grains, as in WT pollen at this stage, but the vacuoles were larger in size (Fig. 15D). The other organelles in the vegetative cell were of similar structure and distribution as in the WT. The generative cell had separated from the intine and contained a nucleus, mitochondria and vacuoles, but no plastids (Fig. 15D).

III.4.2.3. Tricellular pollen stage

At this stage, the tapetum had further degenerated (Fig. 16A) and there were no distinct organelles in the tapetal cells (Fig. 16C). The anther locule was filled with a large quantity of osmiophilic material which may have been released from the degenerating tapetal cells (Fig. 16C and D). In WT microspores, the endintine was wavy at this stage (Fig. 12B), but it was flat in the *ms33* mutant (Fig. 16B). There were more

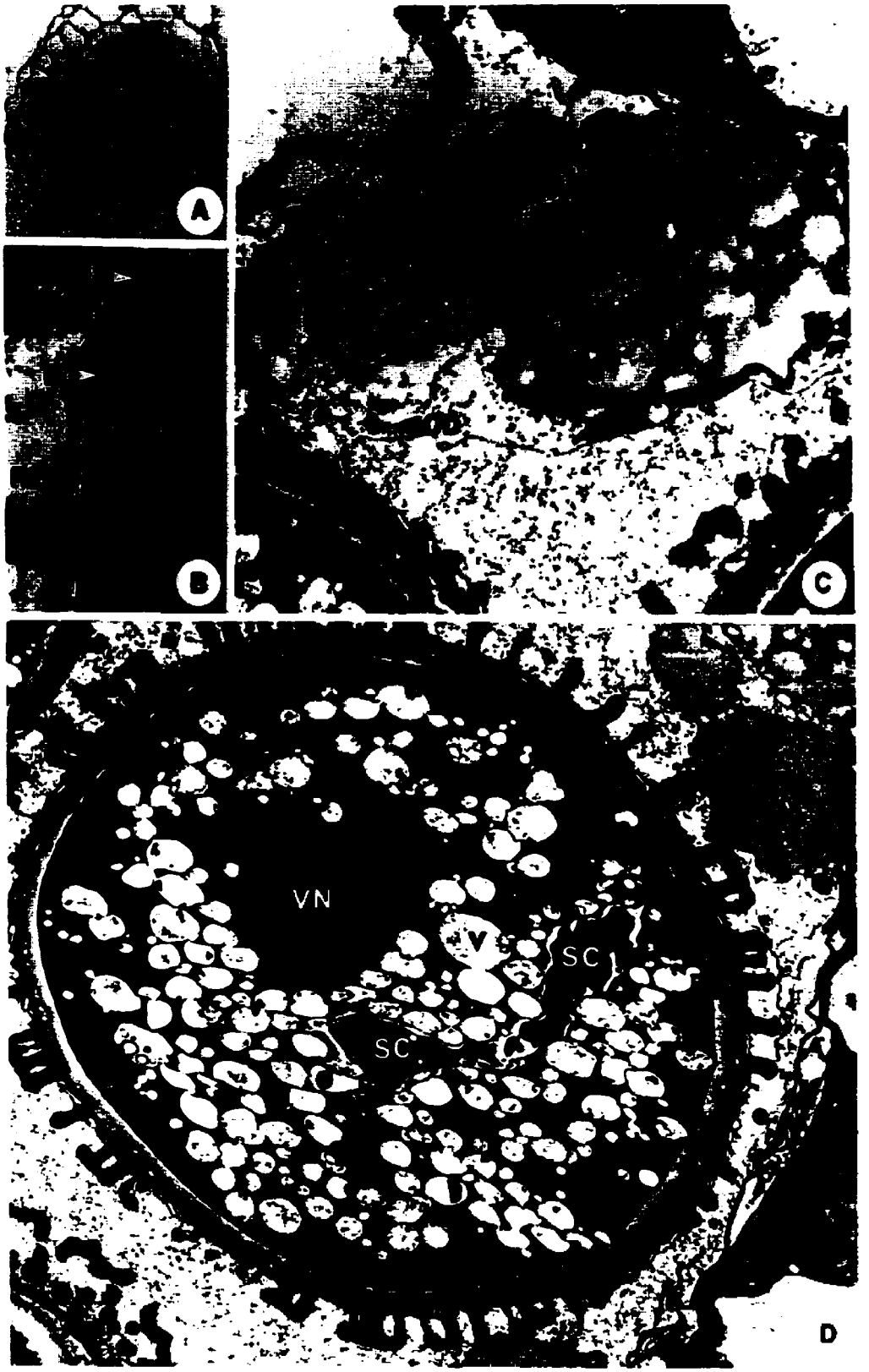
Fig. 15. Bicellular pollen stage in *ms33* anther. A: LM of a cross section of an *ms33* anther (x 420). B: TEM micrograph of a portion of pollen wall (x 21,300). C: TEM micrograph of tapetal cells (x 12,900). D: TEM micrograph of an *ms33* bicellular pollen grain (x 8,200).

Ei = exintine, En = endintine, Ex = exine, GC = generative cell, M = mitochondria, Nu = nucleolus, OD = osmiophilic deposition released from tapetum, P = plastid, R = RER, RE = rectangular electron-lucent deposit, V = vacuole, Ve = vesicle, VN = vegetative nucleus.

A
B
RE
C
M
VB
GC
R
D

Fig. 16. Tricellular pollen stage in *ms33* anthers. A: LM of a cross section of an *ms33* anther (x 490). B: TEM micrograph of a portion of pollen wall (x 27,500). C: TEM micrograph of tapetal tissue (x 7,900). D: TEM micrograph of a tricellular pollen grain (x 6,800).

DT = degenerating tapetal tissue, Ei = exintine, En = endintine, Ex = exine, L = lipid body, OD = osmiophilic deposits released from tapetum, RE = rectangular electron-lucent deposit, SC = sperm cell, V = vacuole, VN = vegetative nucleus.


A
B
C
VN
V
SC
SC
D

osmiophilic structures in the exintine in *ms33* than in WT. The intine in *ms33* at this stage was similar to that in WT at the mature pollen stage (Fig. 13B). There were no differences in the exine in *ms33* and WT. In the vegetative cell the nucleolus was dispersed in the nucleus, and the cytoplasm was full of vacuoles, many of which were fused to each other. Other organelles could not be easily detected in the cytoplasm, but there were some lipid bodies. Two sperm cells, each with a single nucleus, were observed in the pollen grain (Fig. 16D).

III.4.2.4. Mature pollen stage

At the end of pollen development, the tapetal tissue was lacking and an opening was formed in the septum between two locules and later in the stomium region of the anther (Fig. 17A). Mature pollen grains of the mutant were highly vacuolate, and had both large and small vacuoles (Fig. 17A and C). The vegetative nucleus was lobed and without the nucleolus. The cytoplasm of the vegetative cell contained numerous Golgi and lipid bodies (Fig. 17D). Each lipid body, which had an electron-lucent space, was surrounded by a strand of RER. Mitochondria appeared normal and plastids did not contain starch grains (Fig. 17D). The structure of intine was similar to WT at this stage. However, the tryphine appeared to be more homogeneous (Fig. 17B) than that in WT (Fig. 13B).

III.5. Roles of temperature and hormones in the *ms33* mutant

As reviewed in the introduction, almost all types of plant hormones are directly

Fig. 17. Mature pollen stage of *ms33*. A: LM of a cross section of an *ms33* anther (x 680). B: TEM micrograph of a portion of pollen wall (x 16,000). C: TEM micrograph of a mature pollen grain (x 5,100). D: TEM micrograph of cytoplasm of a mature pollen grain (x 26,300).

Ei = exintine, En = endintine, Ex = exine, G = Golgi body, L = lipid body, M = mitochondria, P = plastid, R = RER, Tr = tryphine, V = vacuole, VN = vegetative nucleus.

A
En
Ex
B
C
D

or indirectly involved in the expression of male sterility in higher plants although there is no unique pattern among different species. Based on the phenotype of the *ms33* mutant, i.e., altered flower morphology, the rate of seedling and plant growth, and pollen morphology and development, it is hypothesized that mutation in the *MS33* gene affects one or more endogenous hormones; their levels and/or their signal transduction, which in turn affect the various morphological and developmental traits. This hypothesis was tested by the following sets of experiments. 1. application of plant hormones to whole plants and young inflorescences, 2. hormone treatments to young stamens grown *in vitro* and *in vivo*, 3. the effects of hormones on seed germination, 4. analysis of endogenous hormones, and 5. the construction of *ms33* and hormone-deficient, and hormone-signaling double mutants.

III.5.1. Application of plant hormones

To examine the effects of hormones on plant growth and pollen development in *ms33* and WT plants, various hormones with different concentrations were applied to whole plants as a spray, or as a droplet to young inflorescences with a microsyringe.

Applications of ABA (10^{-5} - 10^{-3} M) and IAA (10^{-5} -10^{-3} M) by both methods, generally inhibited plant growth in both genotypes, and had no apparent effect on pollen development in WT and *ms33* mutant (data not shown). BAP and zeatin (10^{-5} - 10^{-3} M) enhanced plant growth in both *ms33* and WT plants, but these hormones also had no apparent effect on pollen development as well as on stamen filament growth, in WT or the *ms33* mutant (data not shown). GA_1 or GA_3 (10^{-5}-10^{-3} M as a spray) had a strong

influence on the growth of plants. For example, GA_3 (10^{-4} M) increased plant height by approximately 35% in both WT and *ms33* plants, as compared to controls, and induced early flowering in both genotypes (about 4 days earlier than control). The application of GA_3 (5 nmol) to young inflorescences resulted in approximately 20% and 65% increase in stamen filament length in WT and *ms33* respectively (Fig. 18A). The epidermal cell lengths in both genotypes increased by 30% and 85% in WT and *ms33* respectively (Fig. 18B). However, GA_1 or GA_3 application did not restore male fertility in *ms33* plants.

III.5.2. Stamen filament growth *in vivo* in *ms33* and WT flowers

Measurements of filament lengths at different stages of floral bud growth in both *ms33* and WT plants showed that there was no difference in filament growth at early stages of development, i.e., until the bud lengths were about 2.5 mm and filaments were 1.0 mm long (Fig. 19). After this stage, there was a rapid growth of filaments in WT stamens, which coincided with the opening of floral buds, resulting in the final filament length of approximately 2.5 mm (Fig. 18 and 19). In *ms33* flowers, stamen filaments did not show the rapid growth before flower anthesis, and the growth continued at the same steady rate. The final average filament length in the mutant was 1.5 mm (Fig. 19). Measurements of epidermal cells of filaments at various growth stages showed that there was no difference in cell length between WT and *ms33* when the buds were 0.5-0.7 mm, and 2.5 mm in length (Fig. 20). However, in mature flowers, epidermal cells of WT filaments were much longer than those in the *ms33* mutant.

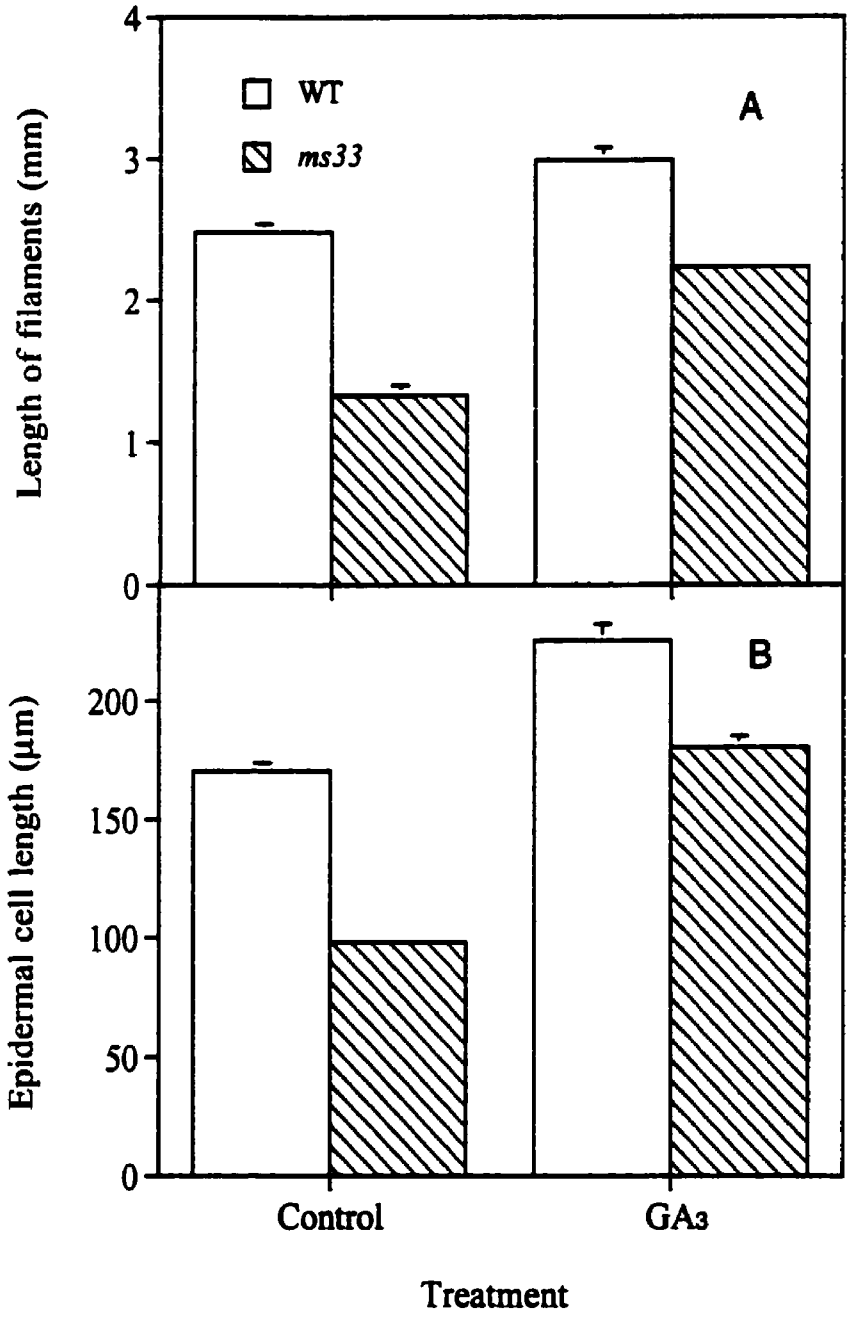


Fig. 18. The lengths of filaments and their epidermal cells in *ms33* and WT flowers treated with GA_3 (5 nmol/inflorescence). 20 filaments and 200 epidermal cells were measured per treatment. Bars indicate S.E.

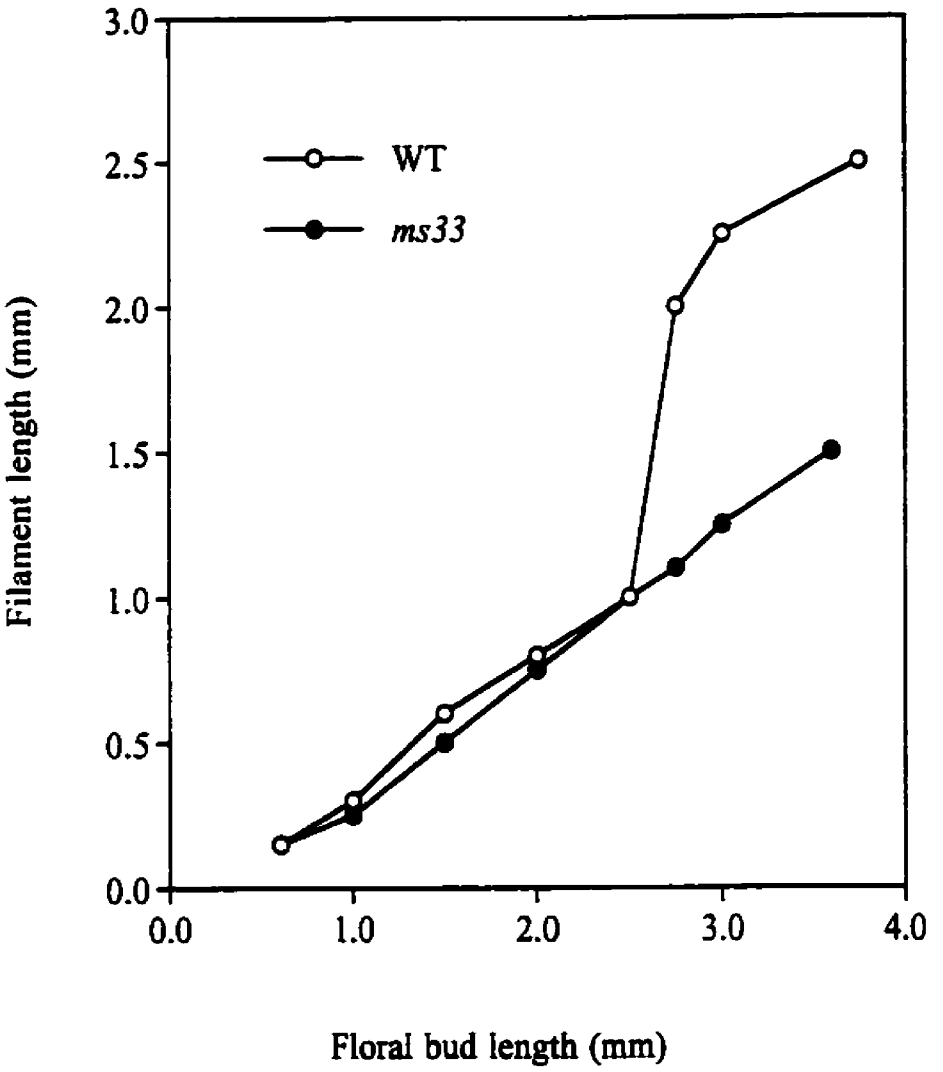


Fig. 19. Filament growth in long stamens of WT and *ms33* floral buds of different sizes. Each value is a mean of 20 filaments from that many flowers.

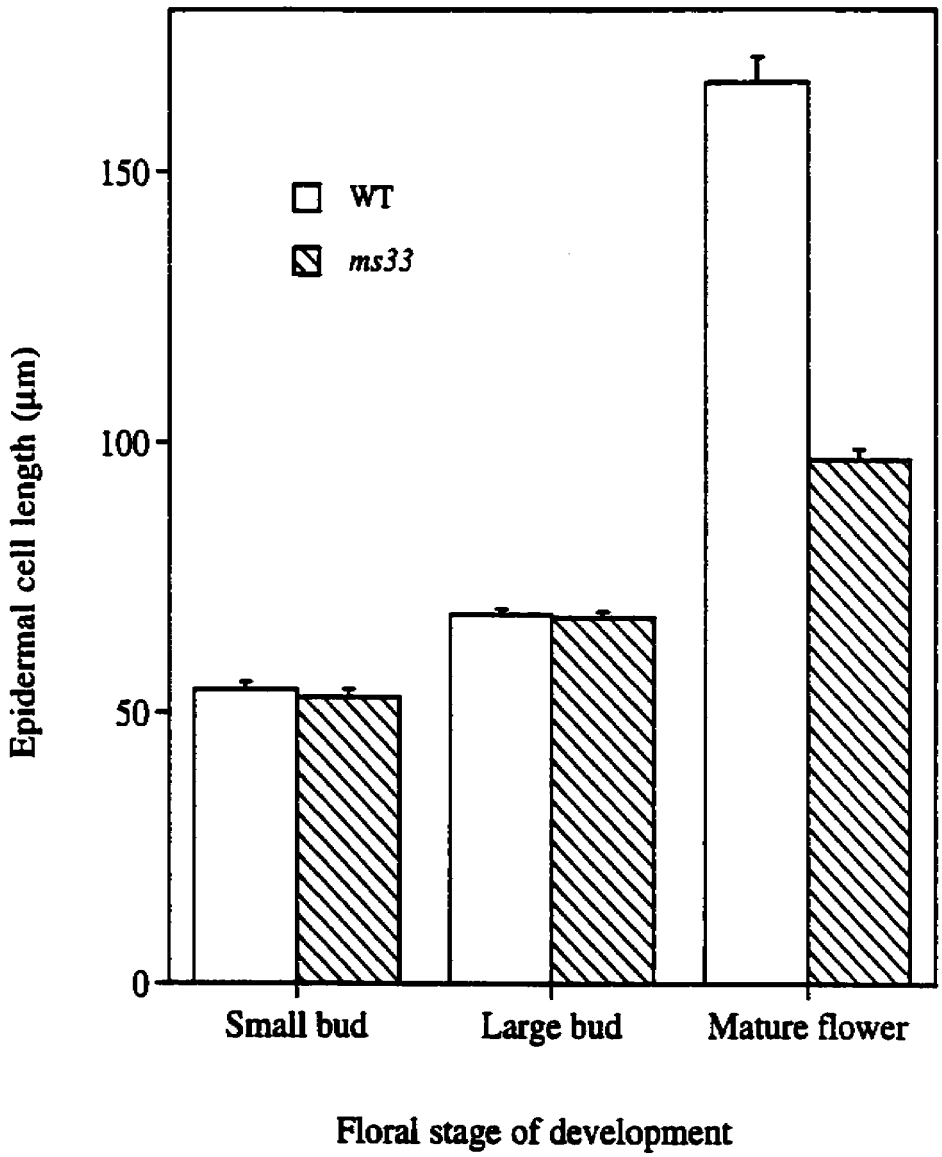


Fig. 20. Epidermal cell lengths of filaments at different developmental stages in *ms33* and WT flowers. 200 cells were measured at each stage. Bars show S.E.
Small bud = 0.5-0.7 mm long; Large bud = 2.5 mm long.

III.5.3. Effects of emasculation and hormonal application on stamen filament growth *in vivo*

Since anthers are believed to have a strong influence on stamen filament growth (Greyson and Tepfer, 1966 and 1967), experiments were conducted in which anthers were removed from WT and *ms33* stamens at an early stage, and their effect on filament growth was examined. In WT stamens, anther removal, when filament length was 0.5-0.8 mm and floral bud length 1.5-2.0 mm, resulted in inhibition of filament growth (Fig. 21A, Table 5). In contrast, the removal of anthers from *ms33* stamens, at the same stage as in WT, enhanced filament growth (Fig. 21B, Table 5). This effect of anther emasculation on filament lengths was correlated with changes in epidermal cell lengths of filaments, i.e., cell lengths of decapitated WT filaments were reduced, but those of *ms33* were increased, as compared to cells in intact stamens (Control) (Table 5).

In WT stamens, the inhibition of filament growth caused by removal of anthers could be reversed by application of IAA or GA_3. IAA treatment completely restored the growth of decapitated filaments and GA_3 significantly increased filament growth (Table 5). Zeatin application did not result in any improvement in decapitated filament growth, but ABA and ethrel further suppressed filament growth compared to the decapitated control. The effects of these plant hormones on filament growth closely paralleled the changes in epidermal cell lengths (Table 5).

In the *ms33* mutant, although the removal of anthers increased filament length, it was still shorter than the WT control (Table 5). When the decapitated *ms33* stamens were treated with GA_3, the filament length was further increased and was similar to the

Fig. 21. Decapitated stamens of WT and *ms33* flowers showing different filament growth. A: WT flower. B: *ms33* flower. In both cases, anthers were removed when the bud length was 1.5-2.0 mm and filament length 0.5-0.8 mm. The flowers were photographed after 4 days of anther removal.

A
B

Table 5. The lengths of filaments and their epidermal cells from *Arabidopsis* WT and *ms33* stamens decapitated and treated with one of the following plant hormones; GA_3 (7 pmol / filament), IAA (14 pmol / filament), zeatin (11 pmol / filament), ABA (9 pmol / filament) or ethrel (17 pmol / filament). Values presented are means ● S.E. of 30 filaments, or 200 cells from 20 filaments per treatment. Different letters in a column indicate significant difference at $P < 0.01$.

Genotype	Treatment	Filament length (mm)	Cell length (μm)
WT	Intact stamen (control)	2.56 ± 0.03^{a}	166.5 ± 1.9^{a}
WT	Decapitated + lanolin cream	2.21 ± 0.05^{b}	136.5 ± 2.1^{b}
WT	Decapitated + GA_3	2.82 ± 0.04^{c}	214.7 ± 2.2^{c}
WT	Decapitated + IAA	2.50 ± 0.03^{a}	186.5 ± 0.5^{d}
WT	Decapitated + zeatin	2.27 ± 0.04^{b}	141.4 ± 1.7^{b}
WT	Decapitated + ABA	1.22 ± 0.06^{d}	81.1 ± 1.1^{e}
WT	Decapitated + ethrel	1.01 ± 0.04^{ej}	95.2 ± 1.2^{f}
ms33	Intact stamen (control)	1.45 ± 0.03^{f}	96.8 ± 0.5^{fh}
ms33	Decapitated + lanolin cream	1.88 ± 0.07^{g}	125.6 ± 1.3^{g}
ms33	Decapitated + GA_3	2.54 ± 0.03^{a}	182.1 ± 2.0^{d}
ms33	Decapitated + IAA	2.14 ± 0.09^{b}	144.6 ± 1.6^{b}
ms33	Decapitated + zeatin	2.00 ± 0.08^{g}	104.9 ± 0.8^{h}
ms33	Decapitated + ABA	1.10 ± 0.02^{dj}	86.1 ± 1.2^{e}
ms33	Decapitated + ethrel	0.84 ± 0.02^{i}	64.8 ± 0.8^{i}

WT control (Table 5). Application of IAA also enhanced filament growth, but it was less than that induced by GA_3. Zeatin did not stimulate the growth of decapitated stamens, and both ABA and ethrel strongly inhibited the growth of filaments (Table 5).

III.5.4. Effects of hormones on stamen filament growth *in vitro*

Stamen filaments are initiated in the 3rd whorl on the floral meristem, i.e., after sepals and petals, and before carpels. Thus, it is possible that the growth of stamen filaments in the flower is affected by other floral organs developing on the meristem. Therefore, the growth of isolated stamens cultured *in vitro* was examined. Young stamens (0.5-0.8 mm in length) were excised from *ms33* and WT flowers at the same stage as that in emasculation experiments and cultured *in vitro* in the MS medium containing different plant hormones. The growth of control isolated WT stamens *in vitro* was less than that of the stamens in intact flowers (Table 5). In contrast, *ms33* stamens grew longer *in vitro* (Table 6). The growth of decapitated WT stamens in culture was inhibited, but that of *ms33* stamens was unaffected, compared to the respective intact stamens (Table 6). GA_3 promoted filament growth of WT intact stamens cultured *in vitro*, but IAA and zeatin had no effect. However, all of the hormones enhanced the growth of decapitated WT filaments, i.e., without anthers. In *ms33*, filament growth of intact stamens was not affected by any of these hormones, but the growth of decapitated filaments was stimulated by GA_3 and IAA, but not by zeatin (Table 6).

Table 6. The filament lengths (mm) of stamens cultured *in vitro* in MS medium with or without (control) one of the following plant hormones; GA_3, IAA and zeatin. Stamen primordia of 0.5-0.8 mm in length were cultured and observations made after 72 hr. Values presented are means ± S.E. of 20 filaments per treatment. Different letters in a column indicate significant difference at $P < 0.05$.

Plant hormone ($mg\ l^{-1}$)	WT		*ms33*	
	With anther	Without anther	With anther	Without anther
Control	2.31 ± 0.08^{a}	1.95 ± 0.09^{a}	2.03 ± 0.10^{a}	1.89 ± 0.05^{a}
GA_3 (1.0)	2.66 ± 0.05^{b}	2.19 ± 0.05^{b}	2.26 ± 0.04^{a}	2.28 ± 0.13^{b}
IAA (1.0)	2.50 ± 0.07^{abc}	2.24 ± 0.06^{bc}	2.14 ± 0.10^{a}	2.19 ± 0.05^{bc}
Zeatin (1.0)	2.39 ± 0.09^{ac}	2.29 ± 0.06^{bc}	2.21 ± 0.11^{a}	1.81 ± 0.09^{a}

III.5.5. Effects of environmental factors on stamen and pollen development

As reviewed in the introduction, stamen and pollen development is influenced by environmental factors in a number of species. Thus, the effects of two environmental factors, photoperiod and temperature, on stamen filament growth and pollen development in WT and *ms33* mutant were examined.

III.5.5.1. Photoperiod

The *ms33* and WT plants were grown in three photoperiods, i.e., 8/16, 16/8 (control) and 20/4 h (day/night) at normal temperatures, i.e., 22/18°C. In the 20/4 h photoperiod, the growth and development of both the WT and *ms33* plants, and the phenotype of flowers were similar to the control. The pollen grains produced in *ms33* flowers were also non-viable. In the 8/16 h photoperiod, the growth of *ms33* and WT plants was strongly inhibited. The vegetative growth period was much extended and the first flower was produced after 2 months of growth. The height of most plants in both genotypes was less than 5 cm after 4 months of growth. However, male sterility/fertility and stamen filament growth in *ms33* and WT was not affected. WT plants produced some number of seeds under this treatment, but there was no silique or seed development in the mutant.

III.5.5.2. Temperature

The *ms33* mutant plants, identified in the F_2 generation after the first flower bud opened, were grown in five different temperature regimes, i.e., 12/10, 15/11, 18/15,

22/18 (control) and 30/24°C (day/night). In WT, the growth of plants was greater at 30/24°C, compared to other temperatures. The maximum plant height was 32 ± 0.4 cm. Pollen viability was not affected in WT flowers in all temperatures tested, and siliques and seeds were produced. In the *ms33* mutant, the effects of different temperatures on plant growth were similar to those in WT. However, the low temperature, i.e., 15/11°C, had different effects on floral organ growth in both genotypes (Fig. 22). In both *ms33* and WT flowers, the growth of sepals was not affected relative to the controls. The petal lengths increased in *ms33* and WT by approximately 15% to 18%, respectively. However, there was a much greater increase in stamen length (74%) in the mutant compared to the WT (10%) (Fig. 22). In contrast, carpel length was reduced in *ms33* by about 8%, but increased in WT flowers by 30%. In the stamen filaments, there was a corresponding increase in epidermal cell length in WT and *ms33* mutant (Fig. 23), indicating that the effect of low temperature on filament growth was mainly contributed by cell elongation. In *ms33* flowers, the elongation of stamens resulted in anthers being in close position to the stigma (Fig. 24B).

At the low temperature regime, there was also a partial reversion of male fertility in the *ms33* mutant as evidenced by the development of some siliques. There was, however, variation in the number of siliques developed on mutant plants (Fig. 24C) compared to the WT (Fig. 24A). Approximately 43% of mutant plants (in a population of 180 plants) produced seeds. The seeds produced were sown at normal temperature conditions, and the plants that developed showed the *ms33* phenotype (Fig. 24D). The low temperature treatment also increased the number of pollen grains per anther in the

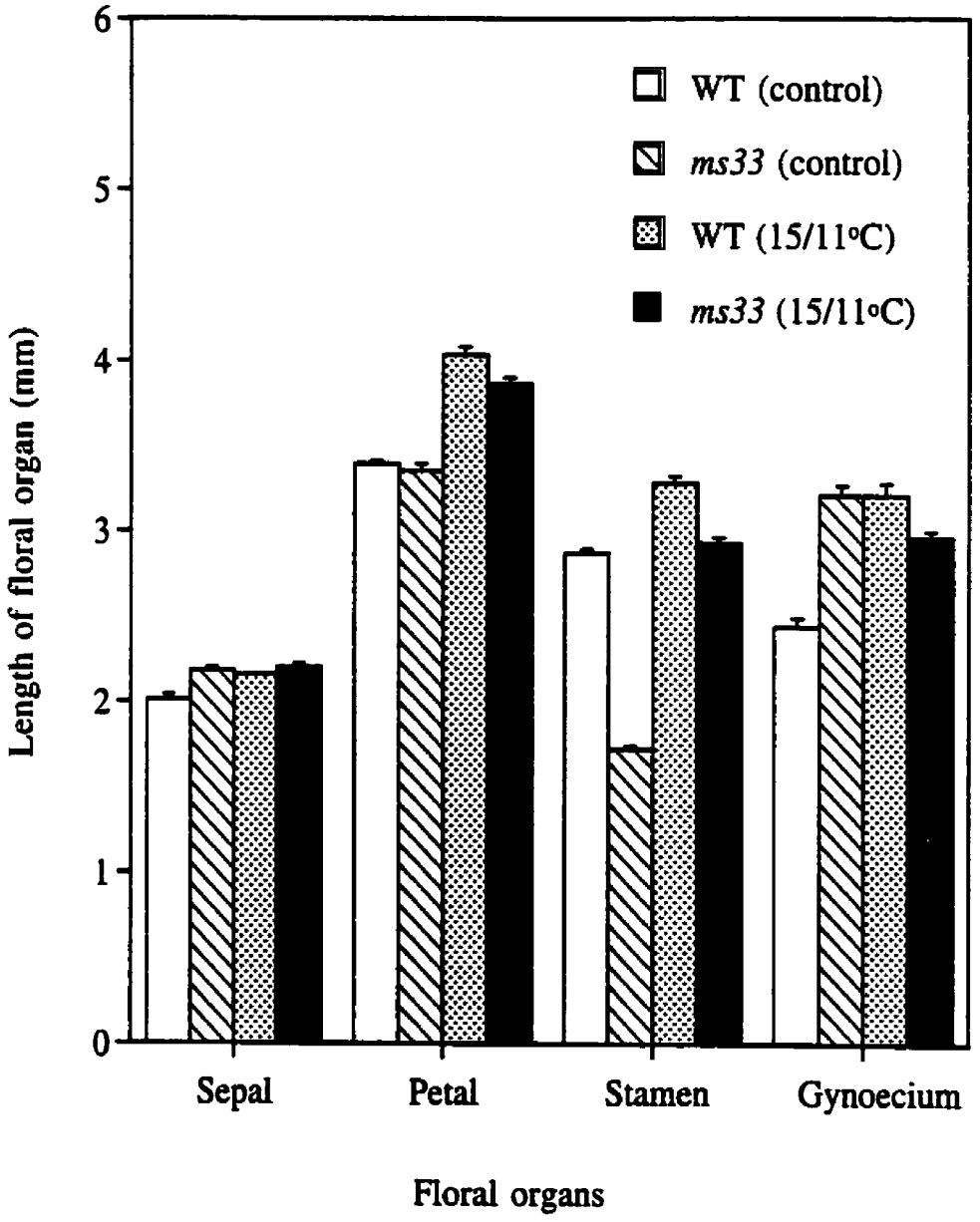


Fig. 22. Lengths of floral organs of *ms33* and WT plants grown in 15/11°C (d/n). Each value is a mean of 20 floral organs from that many flowers. Bars show S.E.

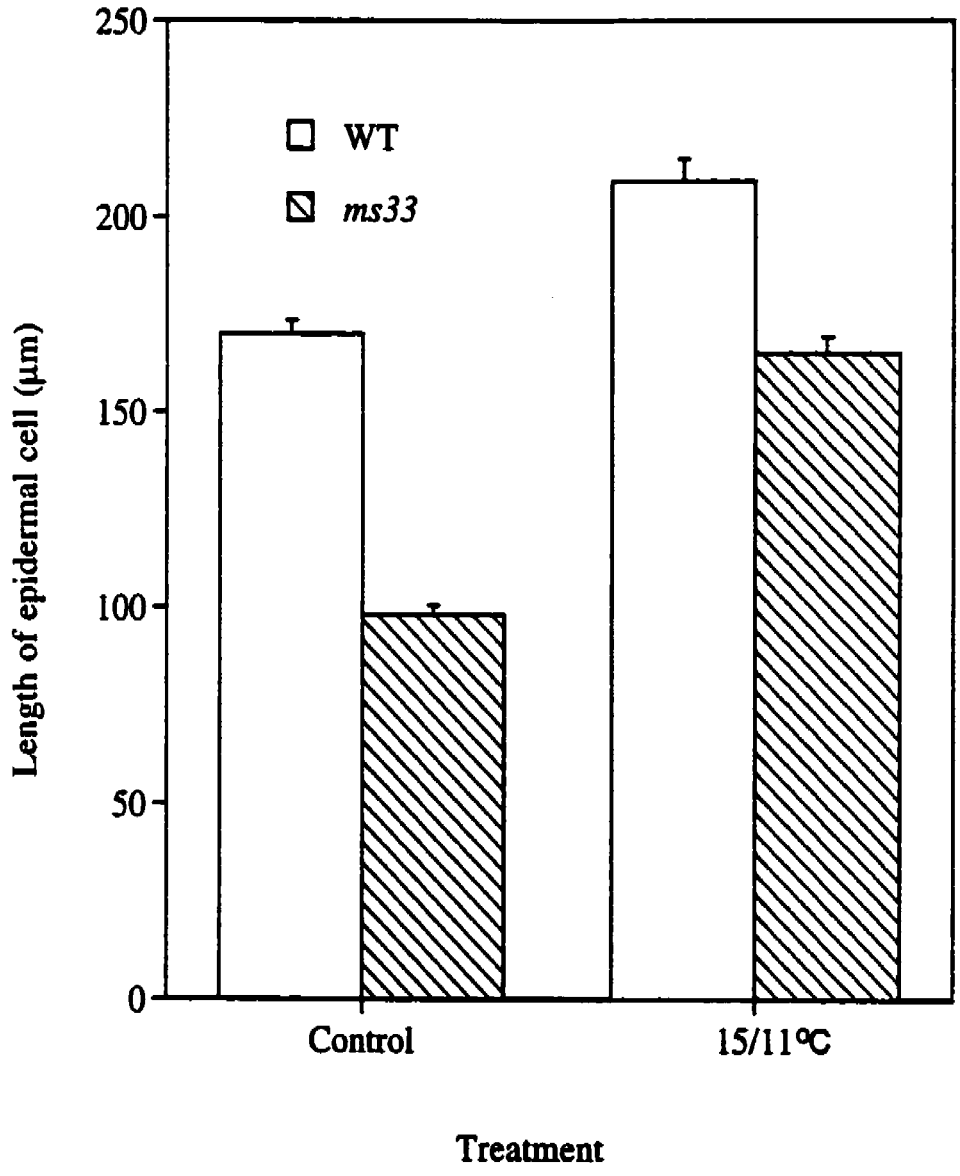
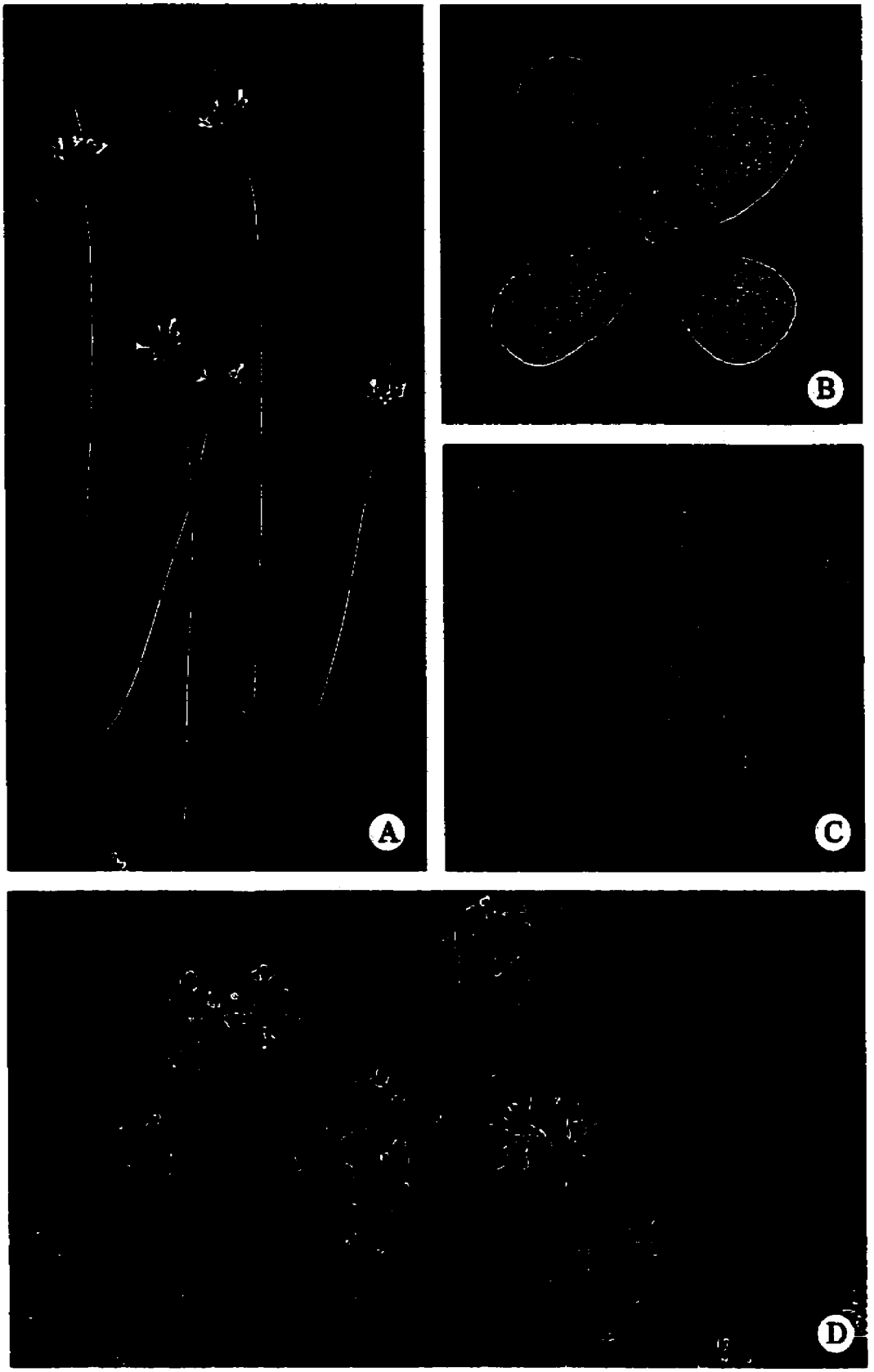


Fig. 23. Lengths of epidermal cells of filaments in *ms33* and WT plants grown in 15/11°C. Each value is a mean of 200 cells. Bars show S.E.

Fig. 24. WT and *ms33* inflorescences grown in low temperature(15/11°C, d/n) with, or without siliques. A: WT inflorescences with a number of developing siliques. B: *ms33* Flower grown in 15/11°C (d/n) showing elongated stamen filaments with anthers at the stigma level. C: Inflorescences of *ms33* plants grown at 15/11°C showing the production of some siliques. D: *ms33* seeds from the treatment with 15/11°C were sown at normal conditions. All plants were male sterile.


A
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ms33 mutant, but it was still less than that in WT anthers at normal or low temperatures (see Table 9).

III.5.6. Seed germination

In section III.3, it was shown that the growth of seedlings and flowering is delayed in *ms33* plants grown at normal temperatures, but, when *ms33* seeds are exposed to 4°C for 3 days, the seedling growth and flowering time in the mutant is similar to the WT (Fig. 5). These observations suggested that seed germination is delayed in the *ms33* mutant at normal temperatures, and that low temperatures overcome this response. To check this possibility, the kinetics of seed germination in *ms33* and WT was examined at different temperatures and light conditions.

In white light at 24°C, WT seeds showed approximately 10% germination after 2 days, and maximum (100%) germination at 4 days (Fig. 25). However, the germination of *ms33* seeds was delayed; germination started after 2 days and maximum germination (98% average) was reached after 7 days. In the dark, germination of both the WT and *ms33* seeds was delayed and maximum germination after 7 days averaged 66% in WT, and 28% in the mutant (Fig. 25). When red light replaced the white light, germination of both the genotypes was similar to the white light treatment, but blue light inhibited germination in both the WT and mutant seeds; more in the latter than in the former (Fig. 26).

Pretreatment of WT and *ms33* seeds either with 15°C or 4°C for 3 days, followed by germination at 24°C in the dark, resulted in enhanced seed germination in both

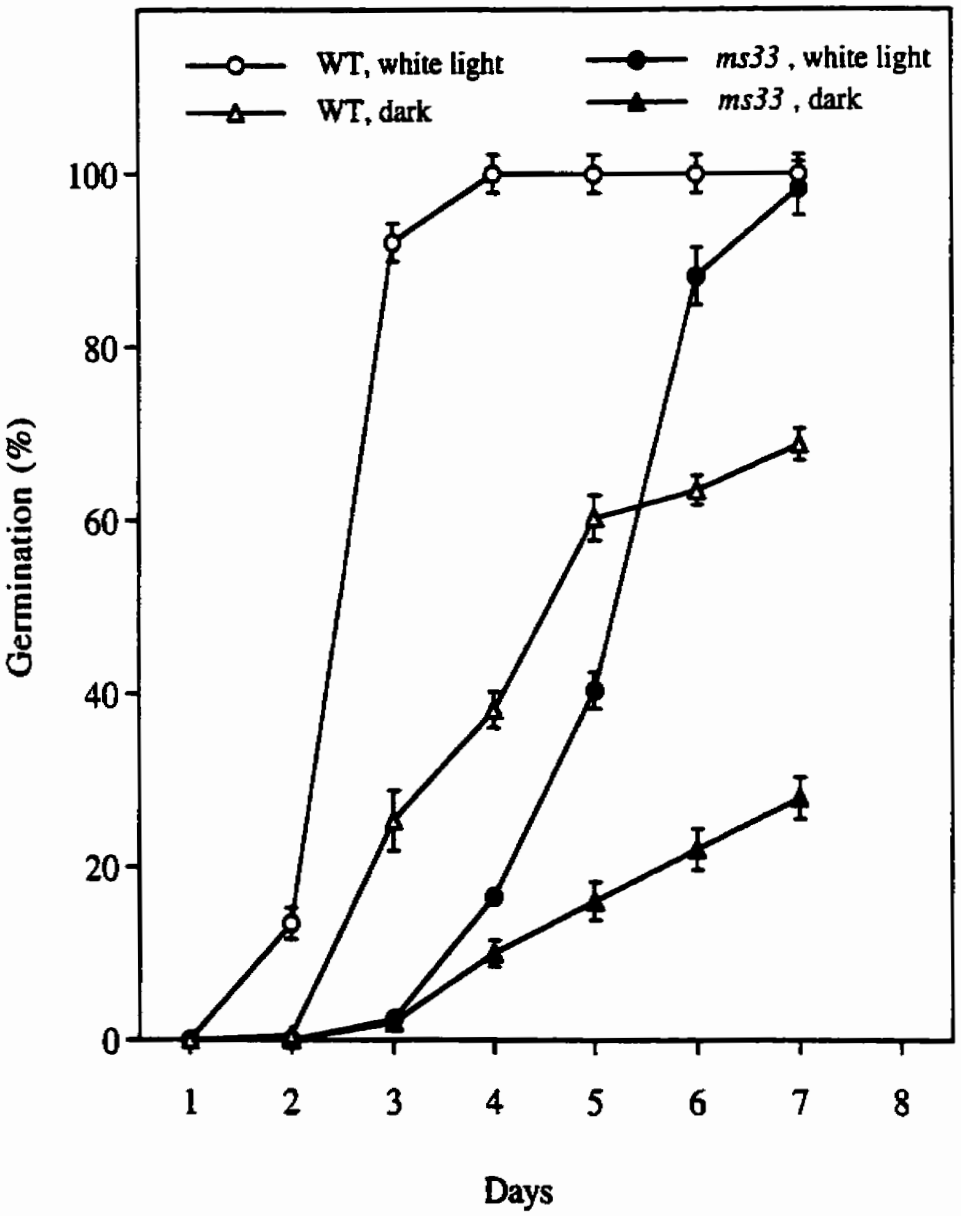

Fig. 25. Germination of *ms33* and WT seeds in white light and in the dark at 24°C for one week. 50 seeds were germinated for each treatment. Each value is a mean of three replicates. Bars indicate S.E.

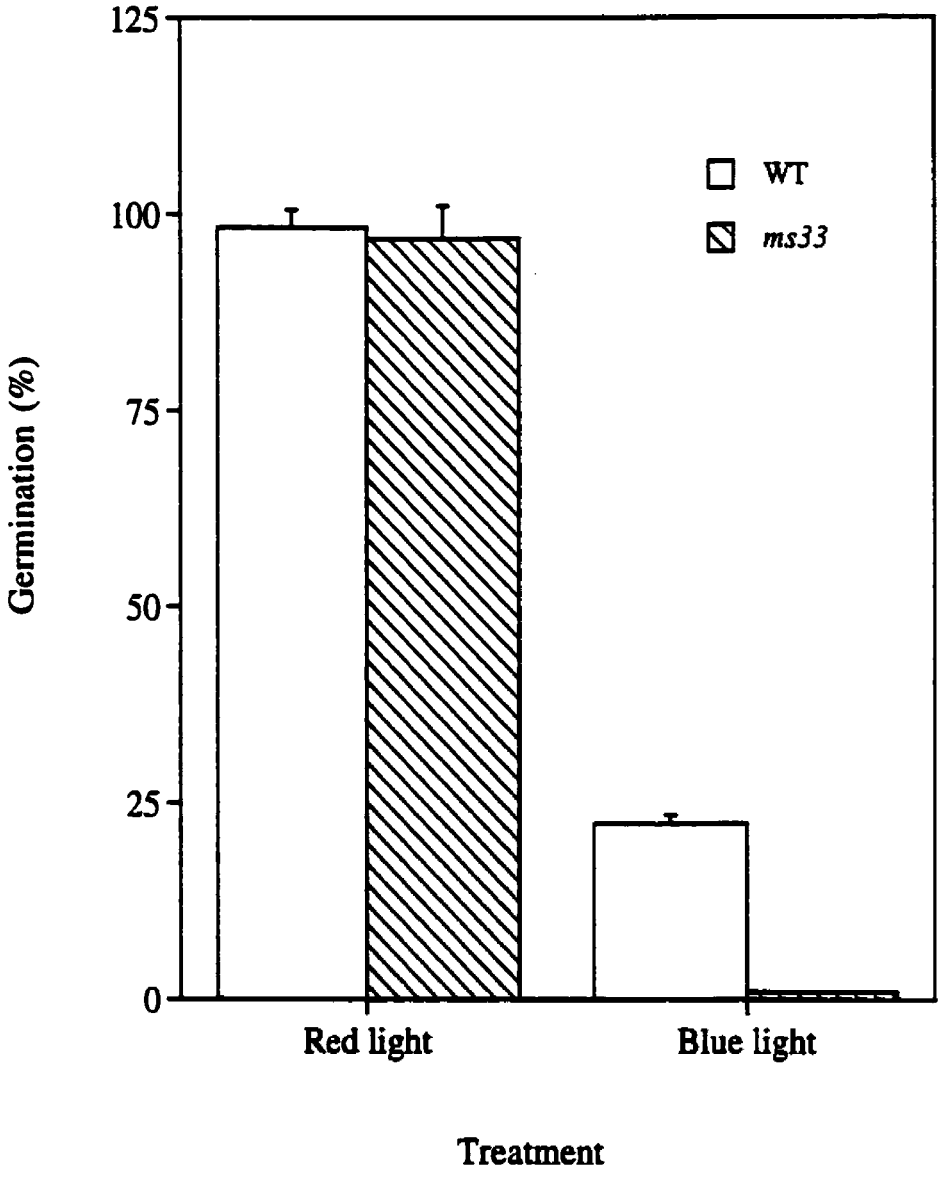

Fig. 26. Germination of *ms33* and WT seeds in red light or blue light after 7 days. 50 seeds were germinated in each treatment. Each value is a mean of three replicates. Bars indicate S.E.

genotypes. WT seeds exposed to 15°C showed approximately 75% germination after 2 days in comparison to nil germination in controls (Fig. 27). Similarly, *ms33* seeds exposed to 15°C showed approximately 55% germination after 2 days versus no germination in normal temperatures. The germination percentage in *ms33* was also increased with 15°C pretreatment and averaged 63% compared to 28% in untreated seeds. Seeds exposed to 4°C showed reduced germination, as compared to 15°C exposure in both WT and *ms33* seeds, but was still higher than at normal (24°C) temperatures (Fig. 27).

As shown in section III.5.1 and III.5.5.2, both low temperature and GAs restore stamen filament growth in the *ms33* mutant (Fig. 18 and 22). Thus, the effect of GAs on WT and *ms33* seed germination in dark was examined because of low germination in dark vs light (Fig. 25). GAs stimulated seed germination in both the WT and *ms33* seeds in the dark. In WT seeds treated with 10^{-4} M GA_3, a small increase in germination was observed compared to the control after one week (Fig. 28). With high GA_3 concentration, i.e., 10^{-3} M, WT seed germination was enhanced and 100% germination was obtained after three days. GA_4 was more efficient in stimulating germination of WT seeds than GA_3 at the same concentration. With 10^{-3} M or 10^{-4} M GA_4, 100% germination was obtained in 2 and 3 days, respectively (Fig. 28).

GAs also stimulated the germination of mutant seeds in the dark; 10^{-4} M GA_3 increased the germination to approximately 40%, and 10^{-3} M GA_3 to 90% after one week, compared to approximately 28% in the control (Fig. 29). With 10^{-3} M GA_4 or 10^{-4} M GA_4, mutant seeds showed 95% germination in 3 and 5 days, respectively (Fig. 29).

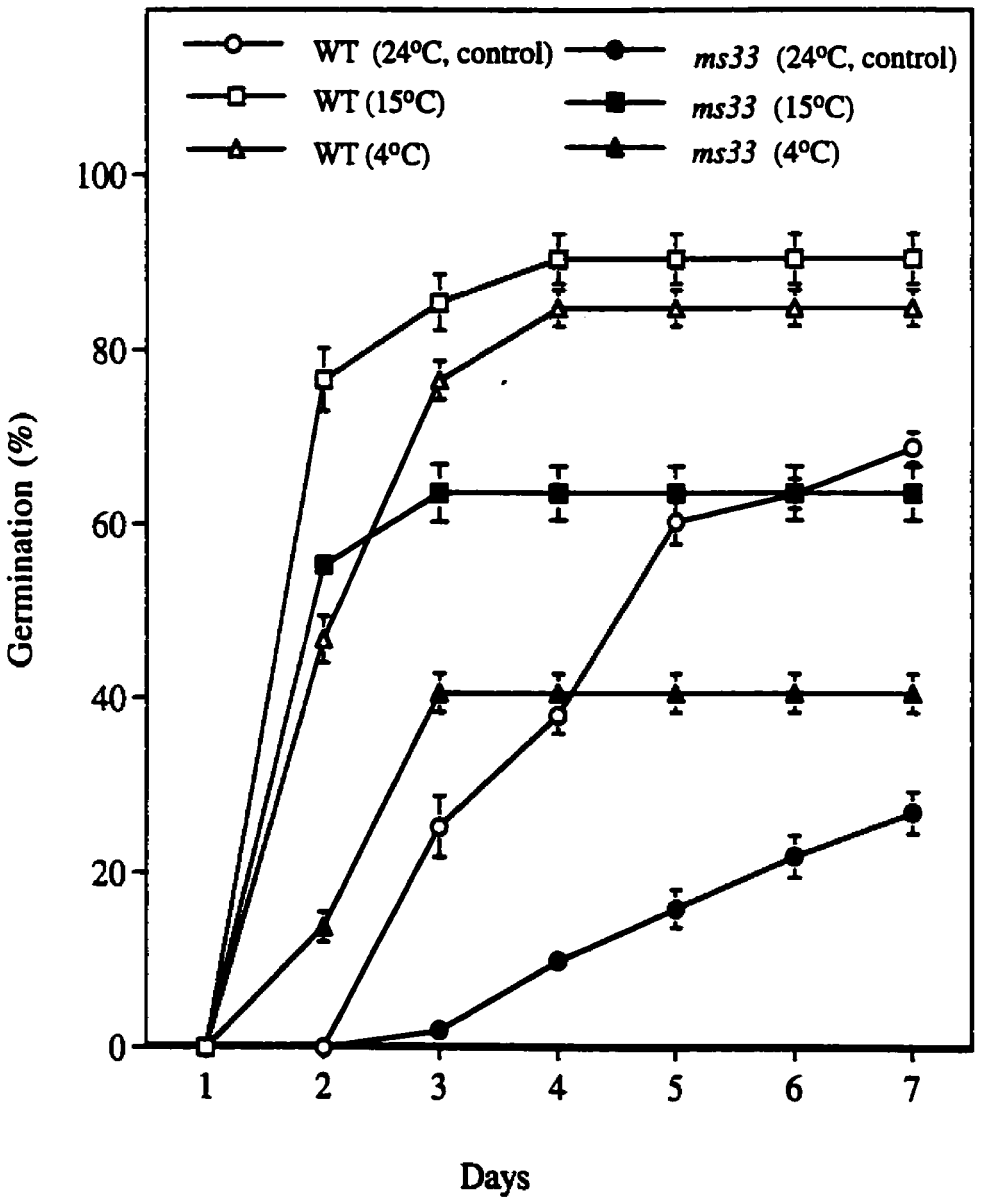

Fig. 27. Germination of *ms33* and WT seeds. Both types of seeds were pretreated either with 15°C or 4°C for 3 days, and then germinated in the dark at 24°C for one week. 50 seeds were germinated for each treatment. Each value is a mean of three replicates. Bars indicate S.E.

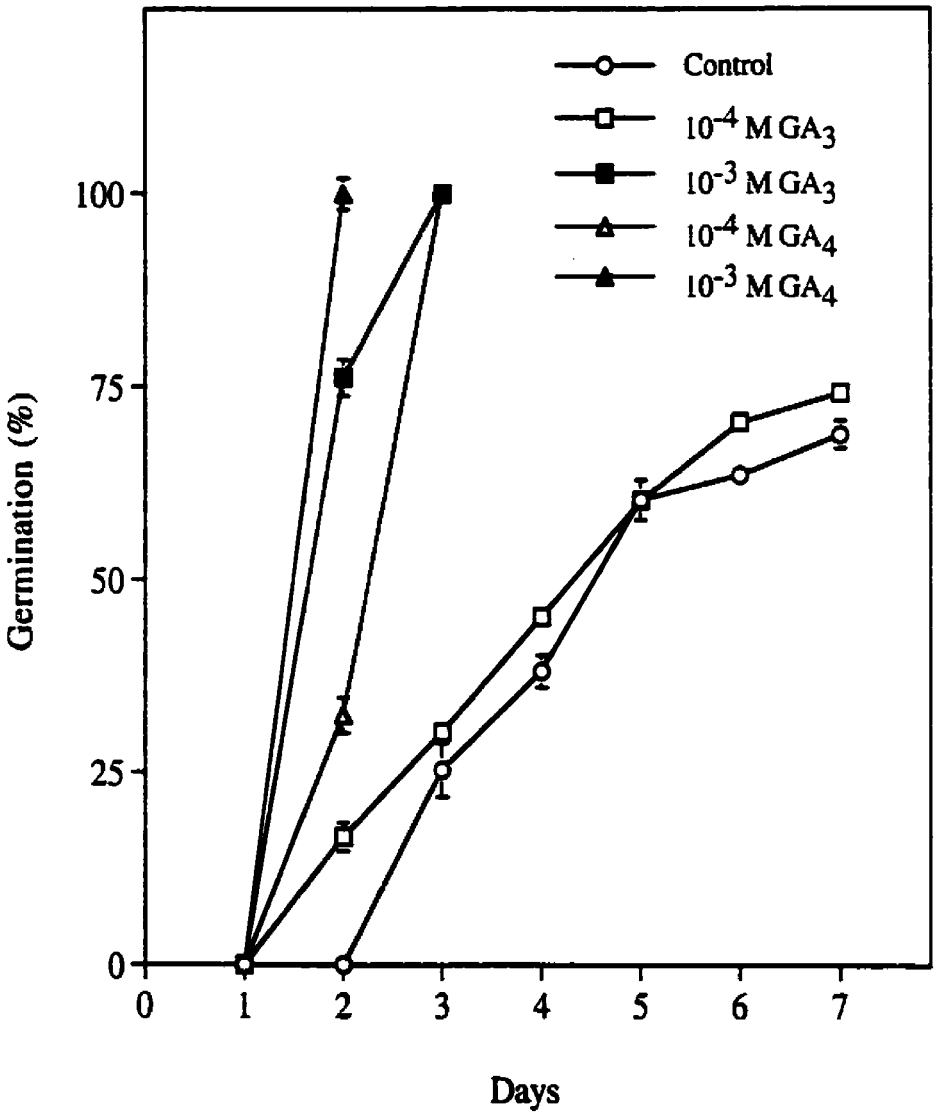

Fig. 28. Germination of WT seeds treated with different concentrations of GA_3 or GA_4 in the dark at 24°C for one week. 50 seeds were germinated for each treatment. Each value is a mean of three replicates. Bars indicate S.E.

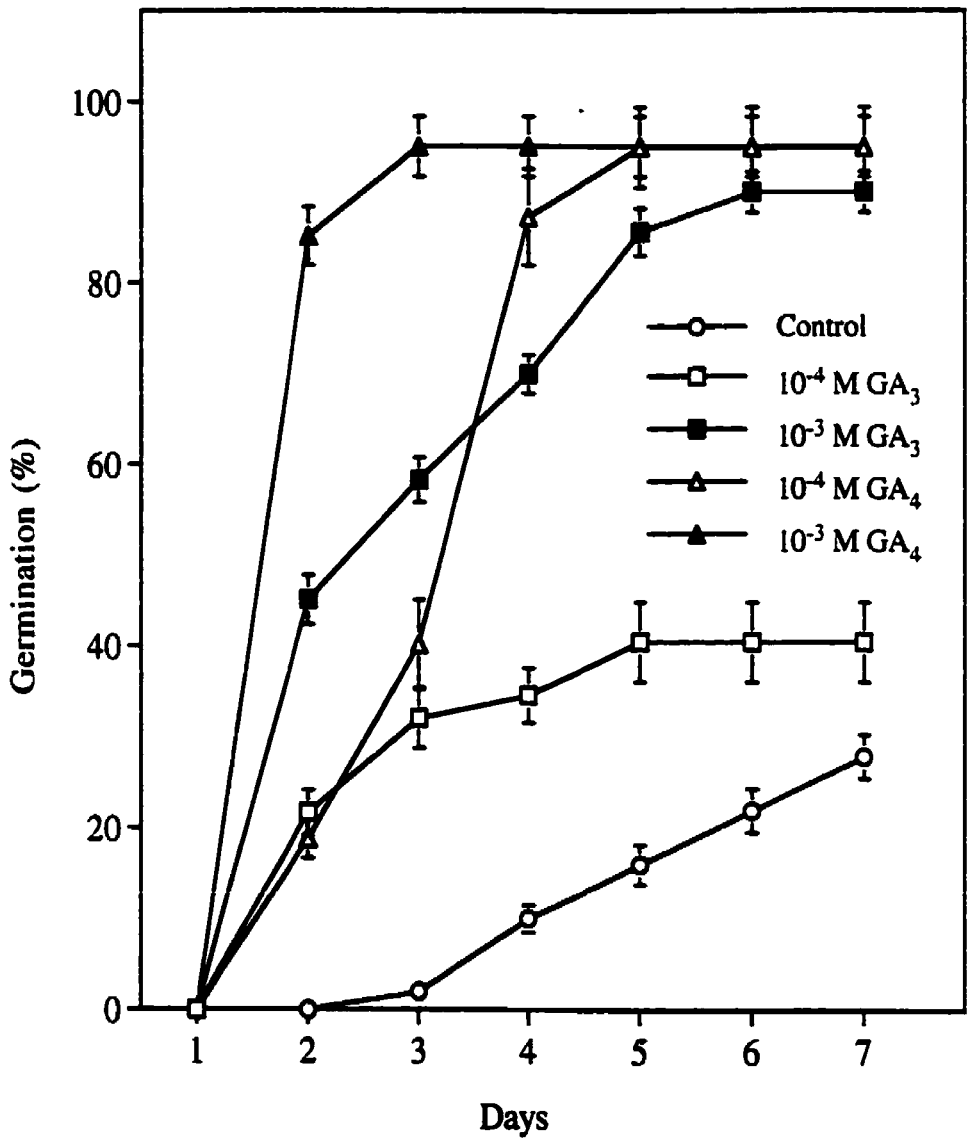

Fig. 29. Germination of *ms33* seeds treated with different concentrations of GA_3 or GA_4 in the dark at 24°C for one week. 50 seeds were germinated for each treatment. Each value is a mean of three repiicates. Bars indicate S.E.

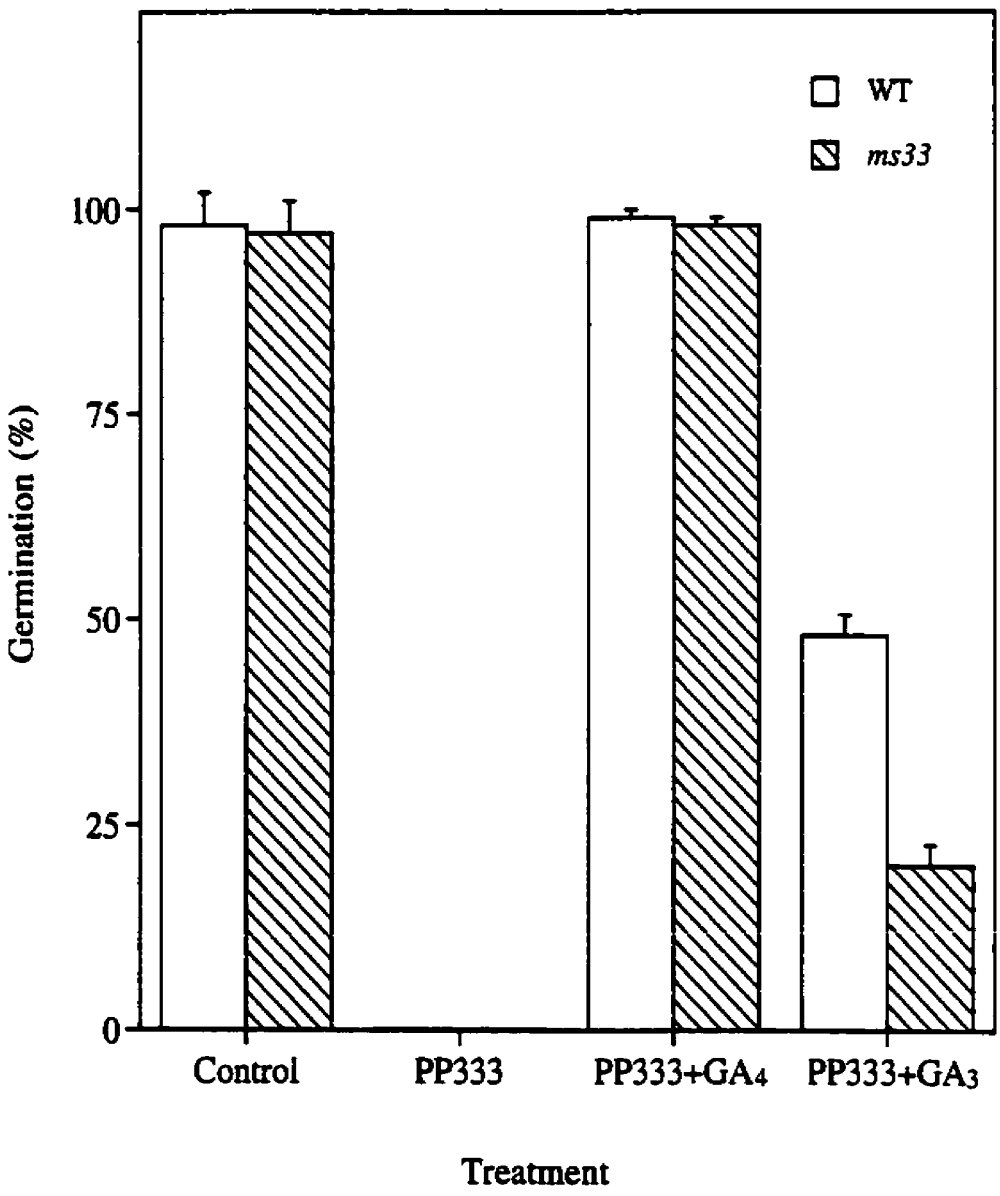

Fig. 30. WT and *ms33* seeds were germinated in H_2O (control), 10^{-4} M PP333, 10^{-4} M PP333 and 10^{-4} M GA_4, or 10^{-4} M PP333 and 10^{-4} M GA_3 in light at 24°C for 7 days. 50 seeds were germinated for each treatment. Each value is a mean of three replicates. Bars indicate S.E.

The germination of WT and *ms33* seeds was totally inhibited in the presence of 10^{-4} M paclobutrazol, an inhibitor of GA biosynthesis, but this inhibition could be entirely overcome by the addition of 10^{-4} M GA_4, and partially by 10^{-4} M GA_3 (Fig. 30).

III.5.7. Effects of GAs on the growth and development of *ms33* and WT plants

Seed germination experiments (III.5.6) have shown that GAs enhance the germination of both *ms33* and WT seeds, and that GA_4 is more effective than GA_3, suggesting that GA_4 may be a major GA affected in the *ms33* mutant. Thus, a comparative study of the effects of GA_3 and GA_4 on the growth and development of *ms33* and WT plants was conducted.

III.5.7.1. Hypocotyl length

The *ms33* and WT seeds were exposed to 15°C in the dark for 3 days to get high rate of germination, and then Petri dishes containing seeds were transferred to a growth chamber set at 24°C and 16/8 h photoperiod. After 24 hrs, germinated seeds with protruding radicles were treated with GA_3 (10^{-5} or 10^{-4} M) or GA_4 (10^{-5} or 10^{-4} M) for 48 hrs, and the hypocotyl lengths of both types of seedlings were measured at the end of the treatment.

The hypocotyl length of *ms33* control seedlings was shorter than that of control WT hypocotyls (Table 7). Both GA_3 and GA_4 stimulated an increase in hypocotyl lengths of *ms33* and WT seedlings, but there was a greater % increase in *ms33*

Table 7. The hypocotyl lengths of *ms33* and WT seedlings grown in different concentrations of GA_3 or GA_4 for 48 hrs. 50 hypocotyls were measured for each treatment. Values presented are means ● S.E.

Treatment	WT (mm)	% increase of WT control	*ms33* (mm)	% increase of *ms33* control
Control	1.46 ± 0.02	-	1.11 ± 0.02	-
GA_3 (10^{-5} M)	1.67 ± 0.02	14	1.56 ● 0.03	41
GA_3 (10^{-4} M)	2.15 ± 0.04	47	2.09 ● 0.02	88
GA_4 (10^{-5} M)	2.20 ● 0.02	51	2.12 ± 0.02	91
GA_4 (10^{-4} M)	2.75 ± 0.03	88	2.64 ● 0.03	138

hypocotyls than in WT. Also, GA_4 induced greater hypocotyl growth than GA_3 at a similar concentration in both *ms33* and WT hypocotyls (Table 7).

III.5.7.2. Plant growth

Plant growth of *ms33* and WT plants was examined *in vitro* by germinating seeds and growing plants in the modified MS medium containing 1.5×10^{-5} M GA_3 or 1.5×10^{-5} M GA_4 for 5 weeks at 22/18°C and 16/8 h photoperiod. Both GA_3 and GA_4 enhanced flowering in *ms33* and WT plants as compared to the controls (Fig. 31). After 5 weeks, control plants of both genotypes were in the vegetative phase, but GA_3- or GA_4-treated plants were flowering. Again, GA_4 was more effective in enhancing reproductive growth than GA_3 (Fig. 31).

III.5.7.3. Pollen number

Young inflorescences, containing 8-10 floral buds, of *ms33* and WT plants were treated two times, at a 3-day interval, with different amounts (5 nmol or 10 nmol/ inflorescence) of GA_3 or GA_4 solution. The pollen number/anther was counted from 10 flowers produced one week after the first treatment. Both GA_3 and GA_4 reduced pollen number in *ms33* and WT anthers, although the inhibitory effect of GA_3 was greater than that of GA_4. There was no effect of low temperature on pollen production in WT plants, but it was increased in the *ms33* mutant, compared to that in normal temperature (Table 8).

Fig. 31. *In vitro* grown *ms33* and WT plants in modified MS medium containing 1.5 x 10^{-5} M GA_3 or GA_4, or without any hormone (control) at 22/18°C and 16/8 h (d/n) photoperiod for 5 weeks.

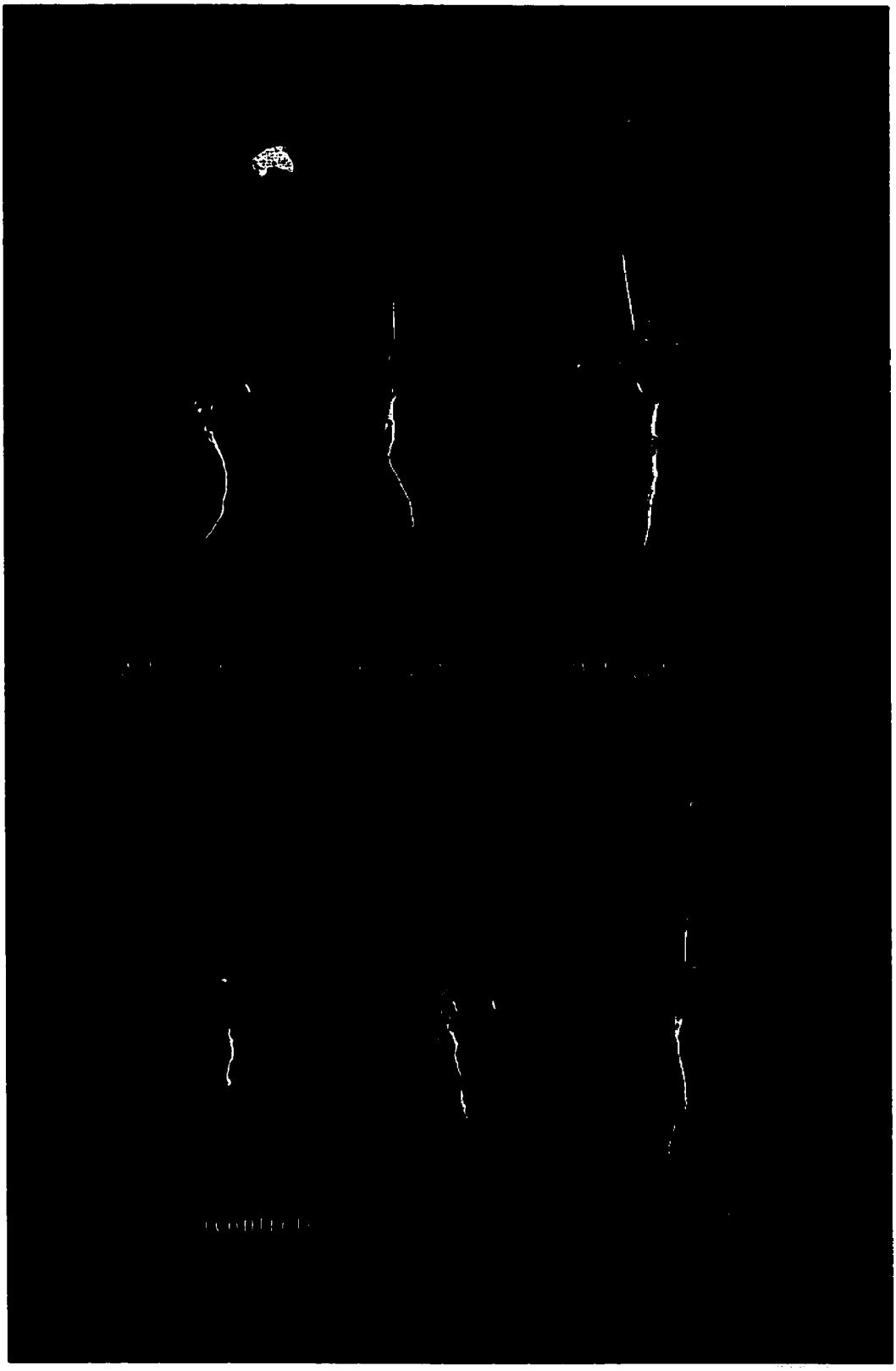

Table 8. Number of pollen grains per anther in *ms33* and WT plants grown in normal temperatures (22/18°C, d/n) and treated with different amounts of GA_3, GA_4 (5 nmol or 10 nmol/ inflorescence), or in plants grown at low temperatures (15/11°C). Each value is a mean ± SE of pollen grains from 10 anthers.

Treatment	WT	*ms33*
Control (22/18°C)	1028 ± 31	592 ± 27
Low temp. (15/11°C)	1044 ± 32	895 ± 32
GA_3 (5 nmol/inflor.)	221 ± 29	106 ± 16
GA_3 (10 nmol/inflor.)	216 ± 21	63 ● 7
GA_4 (5 nmol/inflor.)	562 ± 43	352 ± 32
GA_4 (10 nmol/inflor.)	328 ± 35	309 ± 29

III.5.8. Analyses of endogenous hormones

Experiments with exogenous hormones *in vivo* showed that both GAs and IAA restored stamen filament growth in *ms33* mutant flowers; Zeatin had no effect, and ABA and ethrel suppressed growth. The anther emasculation experiments, and *in vitro* culture of stamens indicated that the filament growth is controlled by the anther as well as by other floral organs. In addition, low temperature treatment enhanced stamen filament growth, increased pollen number and partially restored male fertility in the *ms33* mutant. These observations, coupled with GA-induced restoration of; 1. delayed seed germination, 2. reduced hypocotyl growth, and 3. delayed flowering in the *ms33* mutant, suggest strongly that *ms33* mutation causes a change in GA metabolism in the mutant tissues. In order to determine whether the endogenous hormones are affected in the *ms33* mutant, analyses of GAs, IAA and ABA in the mature flowers of *ms33* and WT plants, grown in normal and low temperatures, were conducted.

III.5.8.1. Endogenous GAs in WT and *ms33* mature flowers

An analysis of GAs showed that in WT flowers the level of GA_4 was much higher than that of other GAs at normal temperatures (Fig. 32), indicating that GA_4 is a major GA in *Arabidopsis* flowers. The analysis also showed that WT flowers contained higher level of total GAs (48.60 ng/g DW) in comparison to *ms33* (19.40 ng/g DW). In particular, the level of GA_4 was much higher (12 times) in WT than that in *ms33* flowers (Fig. 32). However, the level of GA_3 was 2-fold higher in *ms33* than in WT flowers.

At low temperature the levels of all types of GAs declined in WT flowers,

especially that of GA_3 and GA_4; both were reduced 26 and 2.5 times, respectively, compared to the normal temperature. The level of total GAs in WT flowers (20.72 ng/g DW) was similar to that in *ms33* flowers (18.39 ng/g DW) at low temperature, but GA_4 level in *ms33* flowers at low temperature increased by almost two times compared to normal temperatures. The relative amount of GA_3 did not change in the *ms33* flowers at low temperatures and was still higher than that in WT at normal temperatures (Fig. 33).

III.5.8.2. Endogenous IAA and ABA in WT and *ms33* mature flowers

WT flowers contained strikingly high IAA content, approximately 6 times more than the *ms33* flowers (Fig. 34). However, at low temperatures, the IAA content was reduced to less than one-half in WT flowers, but it was still approximately two times higher than that in the mutant flowers at the normal temperature. In the mutant flowers the level of IAA was not affected by low temperature (Fig. 34).

The relative content of ABA in *ms33* flowers was higher than that in WT flowers at normal temperatures (Fig. 35). At low temperature the levels of ABA in WT flowers increased by approximately 55% as compared to the normal temperature, but, there was no change in *ms33* flowers. At low temperature, there was no difference in the level of ABA in *ms33* and WT flowers (Fig. 35).

III.5.9. Construction of double mutants

Analysis of endogenous hormones has shown that *ms33* mutant flowers contain

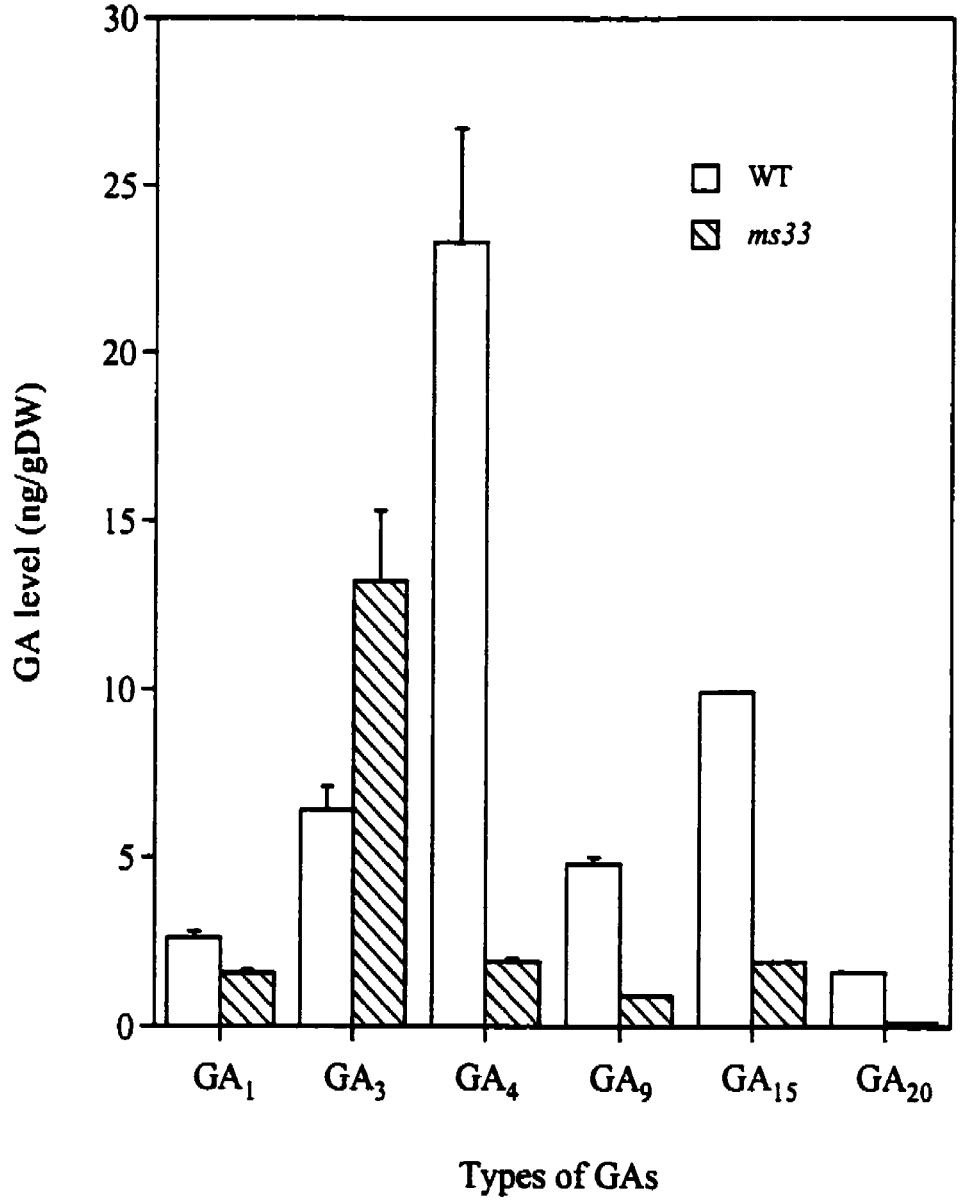

Fig. 32. The levels of endogenous GAs in the mature flowers of *ms33* and WT plants grown at normal temperature (22/18°C). Each value is a mean of two or three replicate samples. Bars indicate S.E.

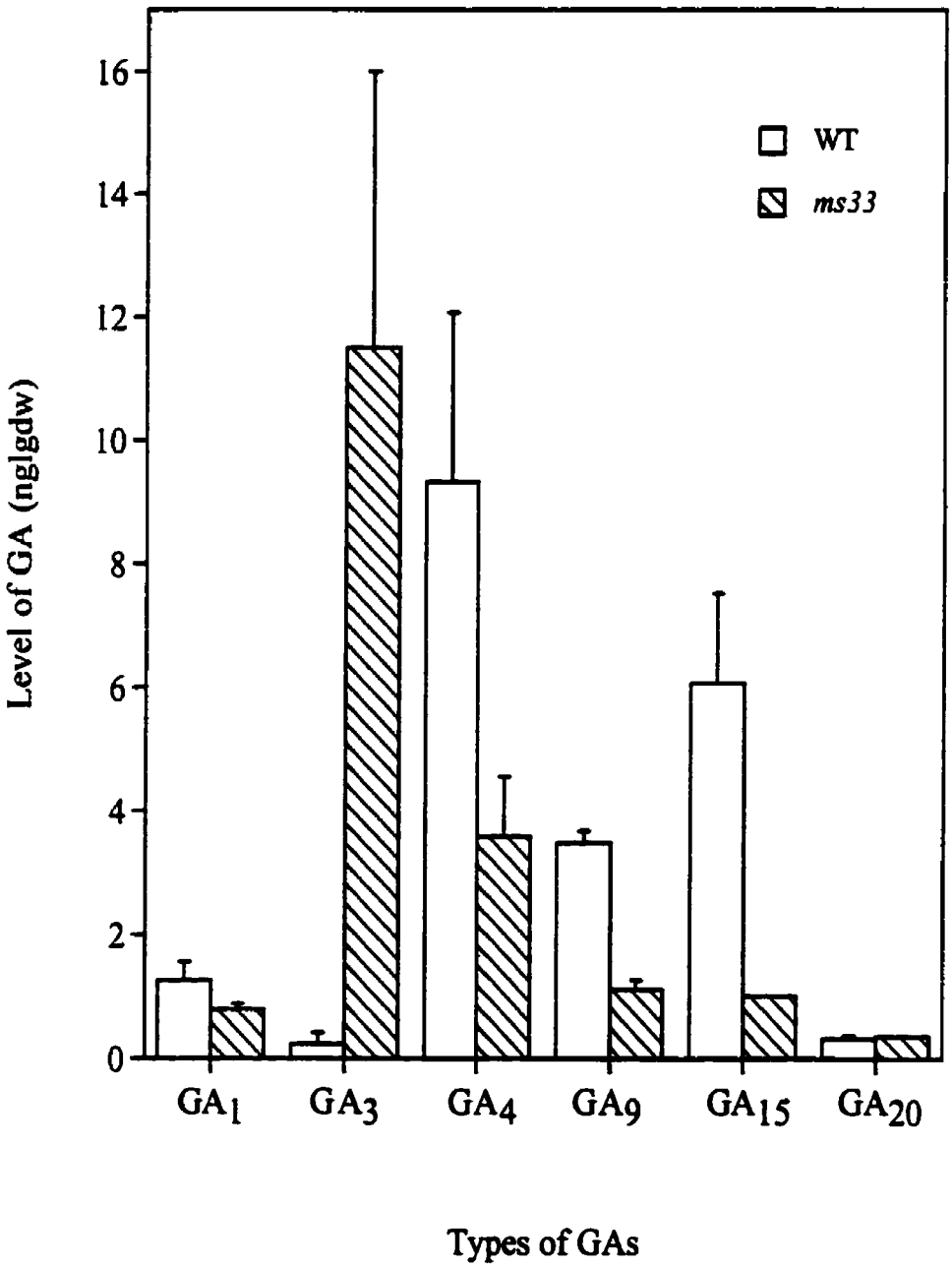

Fig. 33. The levels of endogenous GAs in the mature flowers of *ms33* and WT plants grown at low temperatures (15/11°C). Each value is a mean of two or three replicates. Bars indicate S.E.

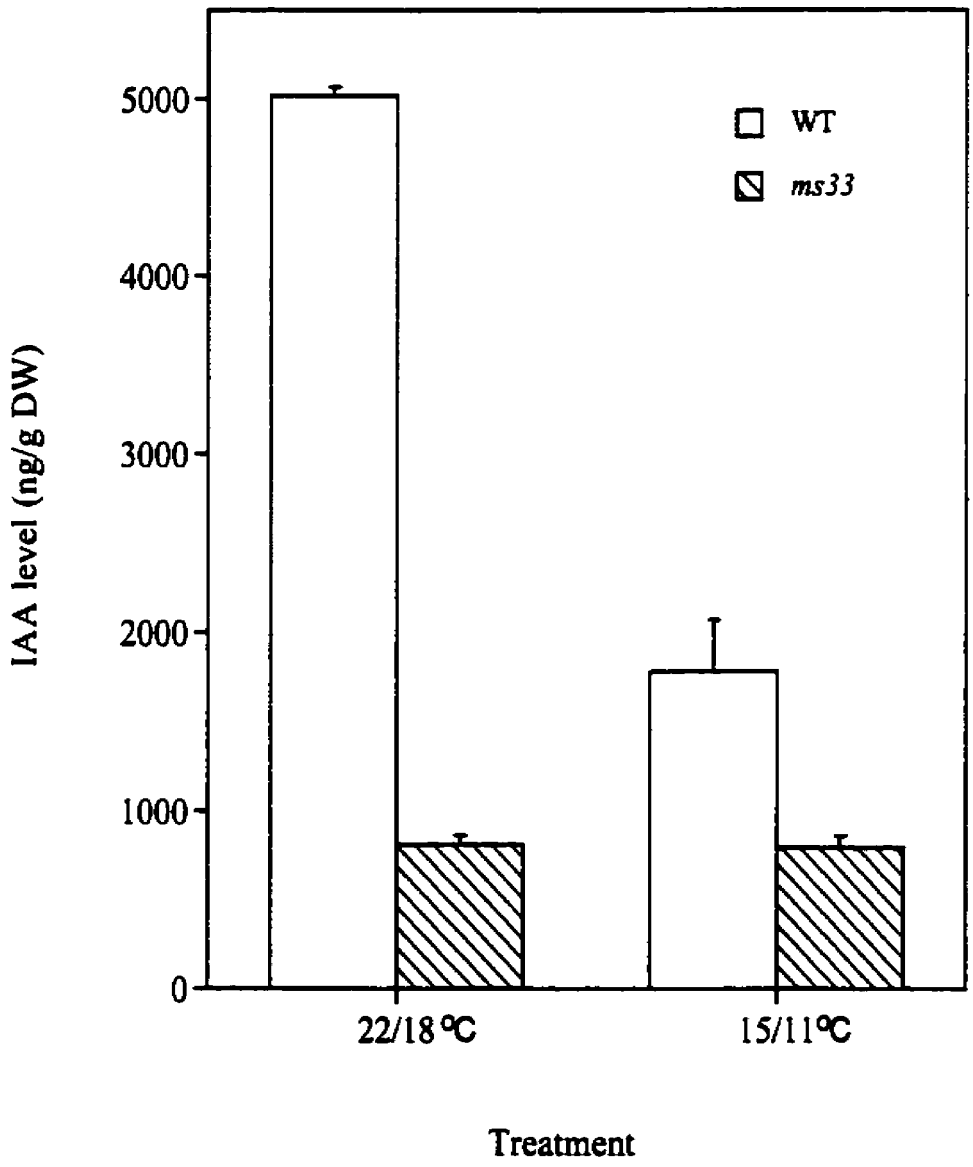

Fig. 34. The levels of endogenous IAA in mature flowers of *ms33* and WT plants grown at different temperatures. Each value is a mean of two or three replicate samples. Bars indicate S.E.

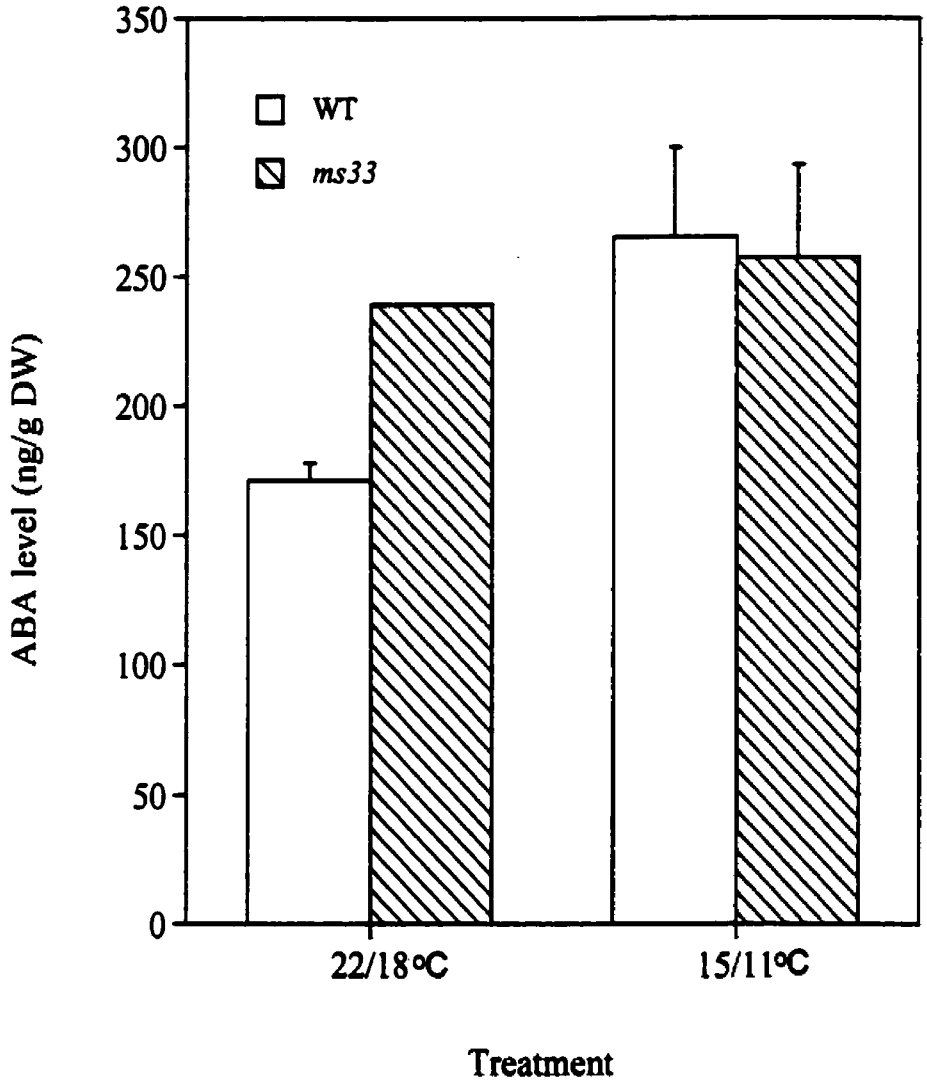

Fig. 35. The levels of endogenous ABA in mature flowers of *ms33* and WT plants grown at different temperatures. Each value is a mean of two or three replicate samples. Bars indicate S.E.

low levels of several GAs, (except GA_3) and IAA, but high ABA content when grown at normal temperatures (Fig. 32). This suggests that the *ms33* mutation likely affects the biosynthesis and/or metabolism of these hormones which in turn leads to the altered growth and development in the mutant. To further investigate the role of hormones in stamen and pollen development, the genetic approach was used by constructing double mutants of *ms33* with two mutants in *Arabidopsis*: 1. an ABA-deficient mutant *aba-1* (Koornneef et al., 1982); and 2. a GA-signal transduction mutant *spindly-3* (*spy-3*) (Jacobsen and Olszewski, 1993). The objective was to determine whether the influence of gene-controlled ABA deficiency, and the activation of the GA signal transduction pathway, would have an effect on *ms33* phenotype.

III.5.9.1. Double mutant *ms33 aba-1*

The *aba-1* mutant has low ABA content because ABA biosynthesis is affected (Koornneef et al., 1982). This mutant is in the Landsberg ecotype background, and is characterized by reduced peduncle length and plant height, an increase in transpiration rate and wilting of plants. Under our growth conditions, the mean height of *aba-1* plants was 7.6 ± 0.5 cm (n = 30 plants), compared to 23.0 ■ 0.2 cm (n = 30 plants) in the WT (see also Fig. 36A and C). The floral phenotype of the *aba-1* mutant was similar to WT, and the flowers were male and female fertile. As described in III.3, the height of *ms33* mutant is similar to WT, but the flowers have short stamens, they produce non-viable pollen, and have a high level of ABA.

The double mutant *ms33 aba-1* was identified in the F_2 progeny. In a total of 923

Fig. 36. Plants of WT, single mutant *ms33* and *aba-1*, and double mutant *ms33 aba-1*.

A: WT, *ms33* and *aba-1* plants grown at normal growth conditions for 5 weeks.

B: Double mutant *ms33 aba-1* flower with shortened stamens. C: Representative plants identified in the F_2 generation after 6 weeks of growth.

WT
ms33
ms1
A
B
C

F_2 plants, four phenotypes were identified (Fig. 36C): 1. 543 tall plants which had long stamens in flowers and normal silique development with seeds (WT), 2. 172 tall plants with short stamens and without silique development (*ms33*), 3. 154 short plants which had long stamens in flowers and had siliques, but were wilting (*aba-1*), and 4. 54 plants of novel additive phenotype that were short, were wilting, had flowers with short stamens, and no silique development (*ms33 aba-1*) (Fig. 36B and C). The ratio of these phenotypes was 9.8 : 3.1 : 2.8 :1 ($X^2 = 3.43$, $P > 0.25$).

III.5.9.2. Double mutant *ms33 spy-3*

spy-3 (Columbia ecotype) is a GA signal transduction mutant. The phenotype of the homozygous recessive *spy-3* plant is similar to the WT plant treated with GAs, i.e., plants exhibit long hypocotyls, elongated peduncles, light green leaves and early flowering (Jacobsen and Olszewski, 1993). Under our normal temperature conditions, the mean height of *spy-3* plants was 52.2 ± 0.4 cm (n = 30 plants), compared to 32.9 ± 0.65 (n = 30 plants) in the WT (Columbia). The height of *ms33* was 24.6 ± 0.3 cm (n = 30 plants) at maturity. All of the floral organs of *spy-3* mutant were also longer than those of WT (Columbia) (Table 9), not reported by Jacobsen and Olszewski (1993).

In 1472 plants of the F_2 progeny from a cross of *ms33* and *spy-3*, four different phenotypes were identified (Fig. 37B). 844 plants were of normal height and produced silique with seeds (WT); 257 plants were of normal height, but flowers had shortened stamens and were male sterile (*ms33*); 286 plants were tall and produced siliques and seeds (*spy-3*); 85 plants were tall with shortened stamens in flowers and were male

Fig. 37. Plants of WT (Landsberg), WT (Columbia), single mutant *ms33* and *spy-3*, and double mutant *ms33 spy-3*. A: Double mutant *ms33 spy-3* flower showing elongated stamens, but they were shorter than the gynoecium. B: Representative plants in the F_2 generation after one month of growth at normal conditions. The double mutant *ms33 spy-3* has an additive phenotype, i.e., tall plant (*spy-3*) and male sterile (*ms33*) and no silique development.


A
B

The last category was novel and showed the additive phenotype of *ms33* and *spy-3* mutations (Fig. 36A and B). The ratio of phenotypes of these plants was 9.8 : 3.0 : 3.3 : 1.0 ($X^2 = 2.36$, $P > 0.25$).

The size of double mutant flowers, in particular petal and carpel length, was larger than either of the *ms33* or *spy-3* mutants, or the WT (Table 9). Sepal length of the double mutant flowers was not different from the single mutants or the WT, but length of the long stamens of *ms33 spy-3* flowers was greater than that in *ms33* mutant, but was similar to the *spy-3* mutant. Thus, with the exception of sepals, the growth of all other organs was increased in *ms33 spy-3* flowers as compared to the *ms33* mutant and the WT (except length of long stamens in Landsberg).

In double mutant *ms33 spy-3* plants grown in low temperatures (15/11°C), partial restoration of male fertility was obtained, similar to the *ms33* mutant grown in low temperatures (Fig. 24C).

Table 9. Lengths (mm) of floral organs of WT (Landsberg ecotype), WT (Columbia ecotype), *ms33* and *spy-3* mutants, and *ms33 spy-3* double mutant. 20 flowers (1 sepal, 1 petal, 1 long stamen and 1 gynoecium/per flower) of each genotype were measured. Values presented are means ● S.E. Different letters in a column indicate significant difference at $P < 0.01$.

Genotype	Sepal	Petal	Long stamen	Gynoecium
WT (Landsberg)	2.04 ± 0.03^{a}	3.39 ± 0.04^{a}	2.87 ± 0.02^{a}	2.44 ± 0.05^{a}
WT (Columbia)	1.83 ± 0.04^{b}	2.94 ± 0.04^{b}	2.45 ± 0.06^{b}	2.40 ± 0.08^{a}
ms33 (Landsberg)	2.18 ± 0.02^{c}	3.35 ± 0.04^{a}	1.72 ± 0.02^{c}	3.21 ± 0.06^{b}
spy-3 (Columbia)	2.04 ± 0.03^{a}	3.48 ± 0.07^{a}	3.14 ± 0.09^{d}	2.77 ± 0.11^{a}
ms33 spy-3	2.02 ± 0.08^{abc}	4.46 ± 0.08^{c}	3.00 ± 0.07^{ad}	3.81 ± 0.08^{d}

IV. DISCUSSION

Male sterility in angiosperms may be expressed at any developmental stage from the initiation of stamens on the floral meristem to the dehiscence of viable pollen (Frankel and Galun, 1977; Kaul, 1988). In many male sterile systems, the expression of sterility is associated with changes in the status of endogenous hormones, and it can also be affected by exogenous hormones (Sawhney and Shukla, 1994). However, conflicting results have been reported in different systems (Sawhney and Shukla, 1994). Thus, the role of hormones in the expression of male sterility remains unclear. In this study, the *male sterile33* (*ms33*) mutant of *Arabidopsis* was used to investigate the possible roles of plant hormones in gene-controlled stamen filament growth and pollen development. The various analyses included; a) morphological characterization of the mutant from seed germination to pollen maturation, b) ultrastructural studies of pollen development, c) exogenous hormone treatments at different stages of plant development, d) the growth of mutant plants under different environmental conditions, e) analysis of three endogenous hormones, and f) construction of double mutants of *ms33* with an ABA-deficient mutant, as well as a GA signal transduction mutant.

The major findings of this study include the following: 1) Mutation in the *MS33* gene not only induces pollen abortion, but also affects the growth of all floral organs, seed germination, seedling growth, and flowering time, 2) The breakdown in *ms33* pollen development occurs during maturation and is associated with the accumulation of

large vacuoles in pollen grains, 3) The stunted stamen filament growth in the mutant is due to the inhibition of cell elongation just before anthesis, and the filament extension can be restored by exogenous GA or IAA, 4) Stamen and pollen development in the *ms33* mutant can be partially restored by low temperature, 5) the relative levels of endogenous hormones, i.e., GAs, IAA and ABA are altered in *ms33* flowers as compared to WT, both under normal and low temperatures, and 6) Double mutant analysis suggests that male sterility in the *ms33* mutant is not related to high ABA levels, or to a possible blockage in the GA signal transduction pathway. Each set of experiments and observations are discussed separately below followed by general conclusions.

IV.1. Phenotypic characteristics of the *ms33* mutant

There were no apparent differences in the morphology of the aerial parts, e.g., leaves and stem between the WT and *ms33* plants grown in normal or low temperature conditions. The earlier description of the *ms33* mutant reported only the inhibition of stamen growth in flowers (Dawson et al., 1993). In our growth conditions, there were several differences between WT and *ms33* mutant flowers. The sepal and gynoecium lengths were greater in the mutant than in the WT (Table 4), and there was a delay in petal growth and in anthesis. The most striking difference, however, was the stamen length. Stamens in *ms33* flowers were much shorter than those in WT. These observations show that the *ms33* mutation affects the growth of all floral organs, and the effect is different on each organ type. This suggests that the *MS33* gene function is

required for the normal growth of all organs. In other male sterile mutants, reduction in the size of floral organs has also been reported, e.g., small size of male sterile flowers in a GMS line in *Brassica napus* (Shukla and Sawhney, 1994), *Cerastium fontanum* and *Spergula pentandra* (Kaul, 1988). However, quantitative data on the size of various floral organs is generally not available, and it is not known whether in other systems genes controlling male fertility affect the growth of all floral organs.

The *ms33* stamens also produced a small amount of pollen that was non-viable. SEM observations showed that these pollen grains were swollen compared to WT pollen (Fig. 4B) and lacked distinct furrows seen on the WT pollen. The large size of pollen may be attributed to the relatively large size vacuoles in *ms33* pollen at maturity (Fig. 17), and the lack of furrows on pollen may be related to the lack of shrinkage associated with pollen desiccation (discussed later). Since female fertility was not affected in the *ms33* mutant, the *MS33* gene is required for male, but not female, gametophyte development. The inability of the shortened stamens to push the anthers to the stigma level and to self-pollinate, has been considered as structural male sterility, and the abortion of pollen development as sporogenous male sterility (Kaul, 1988). Thus, the *ms33* mutant can be regarded as expressing both structural and sporogenous male sterility. A similar situation, i.e., shortened stamens and aberrations in pollen development, has been reported in other genic male sterile systems, e.g. *stamenless-2* (*sl-2*) mutant in tomato (Sawhney and Greyson, 1973) and a GMS line 3-8 in *Brassica napus* (Shukla, 1993).

The *ms33* mutation also causes other pleiotropic effects, i.e., delayed seed

germination, shortened hypocotyl length and delayed flowering. The possible relationship of these developmental defects with changes in endogenous hormones in the mutant is discussed later in section IV.3, 4 and 5.

IV.2. Pollen development in the *ms33* mutant

The *ms33* mutant produces a small amount of pollen which is non-viable as shown by lack of germination *in vitro*, and the inability of pollen to set fruit and seed after manual pollination. Comparative ultrastructural studies on the development of tapetum and pollen revealed that microsporogenesis in the *ms33* mutant was similar to the WT until the vacuolate microspore stage. There were no apparent differences in the cytology of microspores and tapetum before the mitotic division in microspores. However, at the bicellular pollen stage, *ms33* tapetal cells were prematurely degenerated and a large amount of osmiophilic deposits were released into the anther locule that were apparently deposited on the pollen wall (Fig. 15). In addition, there were other abnormalities in the pollen wall and in the cytoplasm of the vegetative cell of *ms33* pollen grains.

First, the process of intine formation was different in *ms33* pollen, and the intine precociously reached the form observed in the mature pollen of WT. In WT pollen at the bicellular stage, the intine consisted of two wavy layers, i.e., thicker exintine and thinner endintine (Fig. 11), but in *ms33* pollen the endintine was much thicker than the exintine at this stage (Fig. 15). The endintine of *ms33* pollen became flat at the tricellular pollen stage (Fig. 16) while WT endintine was still wavy at the same stage, and was flat in

mature pollen (Fig. 12). Intine is a pecto-cellulosic wall layer produced by the vegetative cell cytoplasm (Hess, 1993) which generally begins to form after the completion of the ectexine and adapts to the exine when pollen volume changes during dehydration in the anther (Pacini, 1990a; Marquez et al., 1997). The precocious formation of the intine in *ms33* may influence the dehydration process in the pollen. This is supported by the observations that during pollen maturation, WT pollen at the bicellular stage were engorged with relatively large vacuoles which decreased in size at maturity, presumably as a consequence of desiccation. In contrast, in *ms33* pollen there were numerous small vacuoles after the first mitosis, which later fused to form large vacuoles (Fig. 17C), indicating the lack of, or reduced desiccation. The high vacuolation and large sizes of mature pollen grains in *ms33* also suggests a high water content in the vegetative cell. Pollen desiccation is an important component of pollen maturation and affects pollen viability in many species (Stanley and Linskens, 1974; Barnabás and Kovács, 1997). Thus, the lack of desiccation is likely an important factor affecting pollen viability in *ms33*.

Second, during pollen maturation, the cytoplasm of vegetative cells in WT pollen grains was characterized by many randomly distributed lipid bodies (storage source of energy) surrounded by RER. In *ms33*, however, the number of lipid bodies was much less than that in WT, and there was an electron-lucent space in each lipid body, indicating that they were not fully filled with lipid. In *Arabidopsis*, two storage reserves, i.e., starch and lipid, are present in the vegetative cell during pollen maturation (Zajac, 1997). Starch in plastids is utilized during the maturation process and lipid

bodies are the major reserve in the mature pollen grains (Zajac, 1997). The analysis of acyl carrier protein, a protein involved in lipid synthesis, in *Brassica napus* suggests that pollen lipids are synthesized within the cytoplasm of the vegetative cell. However, the tapetum is also known to provide a large amount of precursors for lipid synthesis (Evans et al., 1992). Thus, the few and not fully filled lipid bodies in *ms33* pollen grains may be related to the early degeneration of the tapetum, i.e., either not enough material was released for lipid synthesis or not released at the correct time.

Third, in the mature WT pollen, there were many electron-lucent globuli in tryphine in the pollen wall (Fig. 13C), but the tryphine in *ms33* pollen was homogeneous (Fig. 17C). Tryphine is produced in the tapetum, mostly in plastids and in other organelles (Weber, 1992). Several functions have been attributed to tryphine, such as protection against water loss, determination of pollen color, retention of pollen aroma, and maintainance of sporophytic proteins in the exine cavities (Dobson, 1988; Pacini and Franchi, 1993). The homogeneous tryphine may restrict water loss from *ms33* pollen contributing to the large size of vacuoles in the pollen. The different structure of tryphine on the *ms33* pollen wall may also be related to abnormalities in tapetum degeneration, since the tapetum releases proteins, exine precursors and pollenkitt or tryphine for deposition on the pollen wall (Pacini et al., 1985).

Based on the evidence outlined above, it is suggested that mutation in the *MS33* gene induces an early degeneration of the tapetal tissue, and that the mistimed release of substances from the tapetum affects the processes of intine formation, tryphine deposition, pollen desiccation, and lipid synthesis in the vegetative cell cytoplasm,

resulting in large non-viable pollen. The tapetum is known to play an essential role in pollen development in terms of providing nutrients, enzymes, proteins, sporopollenin and other substances (Echlin, 1971; Bhandari, 1984; Chapman, 1987; Pacini, 1990b). Aberrations in tapetum development during pollen maturation have been reported in numerous other male sterile systems (see reviews, Edwardson, 1970; Laser and Lersten, 1972; Gottschalk and Kaul, 1974; Bhandari, 1984; Kaul, 1988; Gorman and McCormick, 1997). For example, three different types of abnormal tapetum behavior causing male sterility are reported in *Allium*, i.e., early degeneration, hypertrophy and autolyzation, and delayed degeneration (Holford et al., 1991). The mistiming of tapetum degeneration affecting pollen development also occurs in other species, e.g., premature degeneration in soybean (Palmer et al., 1980; Buntman and Horner, 1983), tomato (Gorman and McCormick, 1997), *Brassica* (Theis and Röbbelen, 1990; Polowick and Sawhney, 1991), and *Arabidopsis* (Dawson et al., 1993), and a delayed breakdown of the tapetum inducing a failure of pollen development in tomato (Polowick and Sawhney, 1995). The role of tapetum in pollen development is further confirmed by transgenic plants containing a construct of a tapetum-specific promoter attached to an RNase or a deacetylase gene, which causes destruction of tapetal cells and induction of male sterility (Mariani et al., 1990; Kriete et al., 1996).

IV.3. *MS33*-controlled stamen filament growth

In WT *Arabidopsis* flowers, the growth of stamen filaments positions the anthers close to the stigma for facilitating self pollination. In the *ms33* mutant, the long stamens

in mature flowers were approximately 40% shorter in length than the WT and barely reached the mid-position of the gynoecium (Fig. 1B). In many species, the major part of filament growth takes place just before flower anthesis (Greyson, 1994). In the WT and *ms33* mutant, filament growth was similar until the buds were approximately 2.5 mm in length, i.e., before the floral buds opened. After this stage, in WT flowers there was rapid growth of filaments, along with petals, and was associated with flower anthesis. Filaments of *ms33* did not show this accelerated growth; instead, growth proceeded at the same steady rate (Fig. 19). This indicates that the mutation in the *MS33* gene controls the rapid elongation of stamen filaments prior to anthesis.

Before the opening of floral buds, the epidermal cell lengths of WT and *ms33* filaments were also not different (Fig. 20), but at maturity the cell length of *ms33* filaments was approximately 40% less than that of WT filaments. This suggests that the absence of rapid filament elongation in *ms33* flowers is due to the inhibition of the final cell-elongation phase of filament growth. Thus, *MS33* seems to affect filament growth by controlling the rapid cell-elongation just before anthesis. The rapid growth of stamen filaments before anthesis and the role of cell elongation in stamen filament growth have been reported in other systems (Schaeverbeke, 1965; Greyson and Tepfer, 1966; Koevenig, 1973; Koning, 1983). However, this is the first report that documents the genetic control of the final cell-elongation phase in stamen filaments.

IV.3.1. Role of low temperature and GAs in filament growth

The stamen filament growth may be affected by external and internal factors and

by other organs in the flower. At low temperatures, the filament growth of both WT and *ms33* stamens was enhanced; however, the increase was much greater in *ms33* than in WT filaments (Fig. 22). The final filament length in *ms33* flowers produced at low temperature was similar to WT filaments at normal temperature (Fig. 22). The increase in filament length in *ms33* and WT by low temperature paralleled an increase in epidermal cell length (Fig. 23), indicating that filament elongation induced by low temperature is mainly due to cell elongation. Thus, low temperature overcomes the cell elongation inhibition caused by the *ms33* mutation. The mechanism(s) by which low temperature modulates the expression of the *ms33* phenotype is not known. One possibility is that low temperature causes a conformational change in the MS33 product, which stimulates cell elongation. This would also explain the small increase in WT epidermal cell and filament length at low temperature.

The stamen filament growth was also enhanced by exogenous GAs. One application of GA_3 resulted in approximately 20 and 65% increase in filament length of WT and *ms33* flowers, respectively (Fig. 17A). Correspondingly, epidermal cell lengths were also increased by 30 and 85% in WT and *ms33* filaments, respectively (Fig. 17B). The increase in epidermal cell length was more than that in filament length and this may be attributed to greater cell length in the mid-region of filaments than at the two end regions. The role of GAs in cell elongation has long been established (Junttila, 1982; Métraux, 1988; Katsumi and Ishida, 1991). Also, GAs were shown to promote stamen filament growth in some, but not all, species (Greyson and Tepfer, 1967; Koevenig 1973; Koning and Raab, 1987). Low temperature is known to increase GA levels in

plant tissues by affecting GA metabolism in many species (Hosoki et al., 1990; Ma et al., 1996; Sim et al., 1996). Recently, Tonkinson et al. (1997) suggested that low temperature increases the sensitivity threshold for GA action in wheat leaf. Thus, the similarity of low temperature and the GA effect on filament growth is not surprising and may be explained as follows. Low temperature alters the MS33 product making it more active in GA biosynthesis or GA signal transduction, which in turn triggers cell elongation.

IV.3.2. Effects of emasculation and hormones on filament growth

Stamens are initiated on the floral meristem, and at the top end the filament connects to the anther. To examine the possible role of the anther (the neighboring tissues) in filament growth, anther emasculation experiments were conducted *in vivo* and *in vitro*. The removal of the anther at the early stages of flower development had an opposite effect on filament growth in WT and *ms33* flowers. Anthers removed from stamens when filaments were 0.5-0.8 mm in length and at a steady growth stage, inhibited filament growth in WT (Fig. 21A), but enhanced growth in *ms33* filaments (Fig. 21B). This growth response paralleled a change in epidermal cell length, i.e., cell length in WT decapitated filaments was reduced, but in *ms33* it was increased, as compared to the respective intact stamens (control) (Table 5). The influence of the anther on filament and cell growth seems to involve translocatable substances, which are produced in the anther and transported to the filament, where they affect cell growth. These substances may be hormonal in nature. Application of plant hormones on

decapitated filaments showed that in WT stamens the major hormones which improved the growth of filaments were GA_3 and IAA. Zeatin had no effect, and ABA and ethrel further suppressed filament growth (Table 5). The effects of these PGSs on filament growth also closely paralleled changes in epidermal cell lengths (Table 5).

In *ms33* stamens, anther removal stimulated filament and cell growth, but the growth was less than that in intact WT stamens (control) (Table 5). Application of GA_3 at the decapitated ends of *ms33* filaments enhanced growth to that comparable in WT control filaments (Table 5). Exogenous IAA also stimulated filament growth, but it was less than that induced by GA_3. Zeatin did not affect growth of decapitated filaments, and ABA and ethrel further inhibited filament growth, i.e., reversed the growth stimulation caused by anther removal (Table 5).

The results from emasculation and hormone application experiments on *ms33* and WT stamens suggest that the anther is an important source of both the inhibitory, i.e., ABA, and promotive, i.e., GAs and IAA, substances that are transported to the filament, where they affect cell elongation. In WT stamens, there may be a relatively high ratio of promoters to inhibitors in the anther, and removal of the anther, therefore, leads to the inhibition of filament growth. In contrast, the *ms33* anthers may contain a higher concentration of inhibitors than promoters, and thus, removal of anthers restores filament growth, although only partially. In WT stamens, growth inhibition by decapitation may also be due, in part, to the wounding of the tissue. The production of ethylene in response to wounding is well known (e.g. Saltveit and Dilley, 1978), and this is consistent with the inhibitory effect of ethrel on the growth of decapitated WT

and *ms33* filaments. In *ms33* stamens, ethylene produced due to wounding may prevent the complete restoration of filament growth in emasculated stamens.

The data from *in vitro* culture of *ms33* and WT stamens showed that the growth of isolated WT stamens was less than that in intact flowers, but that of *ms33* stamens was more than those grown *in vivo* (Table 5, Table 6). This suggests that some promotive substances in WT flowers, and some inhibitory substances in *ms33*, are also supplied by other parts of the flowers to developing stamens. The promotive role of sepals and petals in stamen filament growth was reported in some cases (Koevenig, 1973; Raab and Koning, 1988). However, since the removal of anthers from the isolated stamens inhibited filament growth in WT, but not in *ms33* filaments (Table 6), it suggests that in intact *ms33* flowers other floral organs contribute to the inhibition of filament growth. Stamen primordia cultured in the presence of plant hormones showed that in WT stamens GA_3 enhanced the growth of filaments with or without anthers, IAA and zeatin only affected decapitated filament growth. In *ms33*, GA_3 and IAA only promoted the growth of decapitated filaments, and zeatin had no effect on filament growth with or without anthers (Table 6). These results further support the view that plant hormones, supplied by the anther and other floral organs, are important regulators in *MS33*-controlled filament growth.

Based on the above, the role of plant hormones in *MS33*-controlled stamen filament growth in *Arabidopsis* flowers may be proposed as follows. During the early stage of filament growth, there is a critical balance of growth promoters, i.e., GAs and IAA, and inhibitors, e.g. ABA, which is responsible for the steady growth of filaments.

Before anthesis, i.e., when filaments reach approximately 1.0 mm in length, the *MS33* gene is activated, and the MS33 product regulates an increase in the biosynthesis of the GAs and/or IAA. This temporal increase in the levels of these hormones in WT alters the ratio of promoters to inhibitors, which in turn triggers rapid cell elongation and filament growth. In the *ms33* mutant, the temporal increase in the synthesis of these hormones is blocked and filament growth continues at a steady state (Fig. 19). An alternative explanation is that the MS33 product increases the sensitivity of the filament tissue to either GAs or IAA, or both, thereby enhancing the signaling pathway in cell elongation, but not in the *ms33* mutant.

IV.4. Seed germination

ms33 mutant seeds sown in normal temperatures (22/18°C, d/n) showed delayed seedling growth and late flowering as compared to the WT. But if *ms33* seeds were exposed to low temperature for three days, the growth of mutant plants was similar to the WT (Fig. 5). These observations on plant growth indicated that in the *ms33* mutant seed germination is likely delayed and low temperature overcomes the effect of *ms33* mutation. The kinetic studies of seed germination revealed that in the dark, both the rate of germination and the % germination were less in the *ms33* mutant compared to the WT (Fig. 25). Low temperature enhanced the rate of germination as well as total % germination in both WT and *ms33* seeds, and 15°C treatment was more effective than 4°C (Fig. 27). However, whereas 15°C treatment resulted in approximately 95% germination in WT seeds in the dark, the maximum germination in the mutant was

approximately 65%. Thus, low temperature was unable to completely restore germination in *ms33* seeds. Since low temperature is known to increase endogenous GAs in carnation (Atherton and Harris, 1980), *Thlaspi arvense* (Hazebroek et al., 1993) and apple (Ma et al., 1996), it is possible that the enhanced germination by low temperature may be associated with an increase in endogenous GA levels.

Experiments with exogenous GAs supported the above suggestion. Both GA_3 and GA_4 increased the rate of germination as well as % germination in WT and *ms33* seeds (Fig. 28 and 29). However, with similar GA_3 concentration (10^{-3} M), maximum seed germination in the WT was obtained within 2 days, but in *ms33* it was in 5 days (Fig. 28 and 29). Similar differential response was obtained with GA_4. These observations support the contention that the *ms33* mutant seeds likely have low endogenous GA content. When WT and *ms33* seeds were treated with PP333, an inhibitor of GA biosynthesis, germination was completely inhibited. If both GA_4 and PP333 were present at 10^{-4} M concentration, the germination was the same as control (H_2O). These observations further support the suggestion that low seed germination in the *ms33* mutant is related to reduced endogenous GA levels in the mutant.

The results presented here also showed that GA_4 was more effective than GA_3 on seed germination in both WT and *ms33* mutant. Similarly, the hypocotyl growth was more sensitive to GA_4 than GA_3 (Table 7), and likewise, plant growth and flowering was greatly enhanced by GA_4 in both WT and *ms33* plants (Fig. 31).

Germination response in different light conditions showed that both WT and *ms33* seeds had a higher percentage of germination in white light than in the dark;

however, germination rate in *ms33* was still lower than that in WT. (Fig. 25). The percentage of seed germination in red light was similar to that in white light in both genotypes, but blue light strongly inhibited seed germination (Fig. 26), indicating that phytochromes may mediate red light induced seed germination in *Arabidopsis*. This suggestion is in line with earlier reports that *Arabidopsis* seed is light-requiring, and that the active form of phytochrome is the initial trigger of seed germination (Cone et al., 1985; Koornneef and Karssen, 1994). Our results on seed germination in light, and in the presence of both GA_3 and GA_4 and PP333 (Fig. 30), support the view that light-induction of seed germination in *Arabidopsis* is at least partially dependent on light-induced GA biosynthesis (Karssen and Laçka, 1985; Inoue, 1991; Nambara et al., 1991).

IV.5. Endogenous hormones in the *ms33* mutant and WT flowers

The major phenotypic effect of the *ms33* mutation was the inhibition of stamen filament growth and the production of non-viable pollen. However, these traits could be restored, partially or completely, by hormone application or by low temperature conditions. These observations suggested that the aberrations in stamen and pollen development in the *ms33* mutant may be associated with altered levels of endogenous hormones, and that low temperature effect may be mediated by changes in hormone content. It has been suggested that male sterility is, in part, a manifestation of hormonal imbalance in flowers, particularly in stamens (Durand and Durand, 1991; Singh and Sawhney, 1992; Singh et al., 1992; Shukla and Sawhey, 1994). Thus, analyses of

endogenous GAs, IAA and ABA, the hormones which had an effect on *ms33* mutant filament growth, were conducted in the mature flowers of *ms33* and WT, grown in normal and low temperatures.

IV.5.1. Gibberellins

The data on endogenous GAs clearly showed that there were large differences in GA levels between WT and *ms33* flowers. WT flowers had 12 times more GA_4 than *ms33* flowers. In contrast, a high level of GA_3 occurred in *ms33* flowers (Fig. 32). The total level of GAs was, however, higher in WT than in *ms33* flowers. Relatively high levels of GAs have been associated with flower development, and in particular stamen development, in several species (reviewed in Pharis and King, 1985; Sawhney and Shukla, 1994). In *Mirabilis*, for example, stamens, especially the anthers, had the largest quantity of GAs during flower development (Murakami, 1975). High levels of GAs were also found in both petals and stamens of some other species (Jeffcoat et al., 1969; Sircar et al., 1970; Dathe et al., 1980). Murakami (1973) reported that in *Pharbitis nil*, GA level was high during petal and stamen development and it declined sharply before growth ceased. Thus, increase in GA levels in both petals and stamens may be associated with the growth of these organs.

The major type of active GA varies in different species. GA_3 was the major GA in *Avena* inflorescences (Kaufman et al., 1976), GA_9 in the capitulum of *Chrysanthemum* (Parups, 1980), GA_{19} in rice ear (Suzuki et al., 1981) and GA_{20} in *Vicia* flowers (Dathe and Sembdner, 1980). Exogenous treatments have shown that GA_3

is the most effective GA in stamen development in *Cannabis* and *Luffa* (Mohan Ram and Jaiswal, 1974; Krishnamoorthy, 1972), and GA_4 and $GA_{4/7}$ were very effective in promoting late stages of inflorescence development in tomato (Kinet et al., 1978). Thus, different GAs are associated with flower development in different species, and at different stages of development. In *Arabidopsis* flowers, GA_4 was the major GA (Fig. 32) and was also more effective than GA_3 in enhancing seed germination (Fig. 28 and 29), hypocotyl (Table 7) and plant growth and flowering (Fig. 31) in both WT and *ms33* mutant.

Some male sterile mutants are known to contain low levels of GAs compared to their WT counterparts (Sawhney, 1974; Nakajima et al., 1991). In rice, a male sterile mutant was especially low in GA_4 and GA_1 (Nakajima et al., 1991). Further, some GA-deficient mutants are also male sterile (Koornneef and van der Veen, 1980; Nester and Zeevaart, 1988). In a male sterile mutant in tomato, there is low amylolytic activity in stamens suggesting low GAs and, therefore, aberrations in pollen development (Bhadula and Sawhney, 1989). Moreover, exogenous GAs are known to induce fertility in male sterile mutants in tomato (Sawhney and Greyson, 1973; Schmidt and Schmidt, 1981) and in barley (Kasembe, 1967). Thus, there is a good correlation of low endogenous GAs with male sterility. On the other hand, male sterility can be induced by GA application. For example, male sterility was induced by GA_3 in normal plants of *Zea mays* (Hansen et al., 1976; Krishnamoorthy and Talukdar, 1976), onion (Meer and Bennecom, 1973), pepper (Kohli et al., 1981; Sawhney, 1981) and lettuce (Eeninck and Vereijken, 1978). It is therefore interesting to note that in *ms33* flowers, there was a

high level of GA_3 although the total GA content was low in the mutant as compared to WT. Thus, an increased GA_3 concentration can be related with male sterility in the *ms33* mutant.

The high level of GA_3 and low level of GA_4 in *ms33* flowers suggest the possibility that the *ms33* mutation may affect the interconversion of GAs. In *Arabidopsis*, there are two branches of GA biosynthetic pathways from GA_{12}, the first GA in the GA biosynthesis in plants (Fig. 38, see also Finkelstein and Zeevaart, 1994; Sponsel, 1995). 1) The early 13-hydroxylation of GA_{12} to form GA_{53}, GA_{44}, GA_{19}, GA_{20}, GA_5 and GA_3. 2) The non-13-hydroxylation of GA_{12} to form GA_{15}, GA_{24}, GA_9 and GA_4 (Fig. 38). GA_3 is one of the products of the early 13-hydroxylation pathway, and GA_4 is synthesized from the non-13-hydroxylation pathway. Based on our results, it appears that the MS33 product stimulates the non-13-hydroxylation pathway of GA biosynthesis leading to high concentration of GA_{15}, GA_9 and GA_4, as shown by the endogenous analysis data (Fig. 32). Conversely, in the *ms33* mutant, the early 13-hydroxylation pathway is favored resulting in greater accumulation of GA_3. Both pathways are known to occur in *Arabidopsis* plants (Talón et al., 1990).

IV.5.2. Indole-3-acetic acid

Application of IAA to emasculated stamens, and the isolated stamens cultured *in vitro* in the presence of IAA, showed enhanced stamen filament growth in *ms33* flowers (Table 5 and 6). Thus, it seems that the mutant stamens may also be deficient in endogenous IAA, and that anthers may be the source of this auxin. Since petal

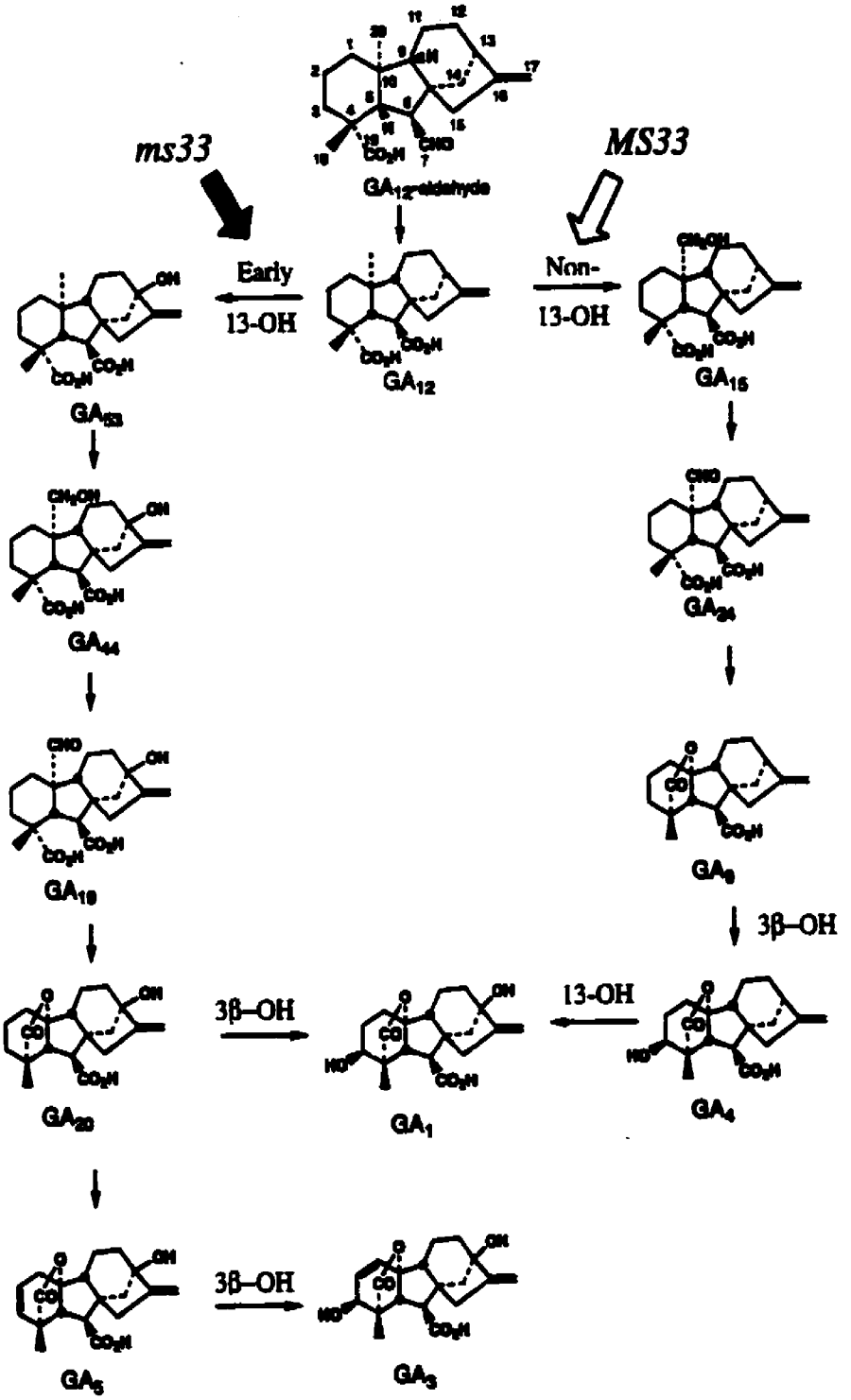

Fig. 38. A portion of biosynthetic pathways of gibberellins in *Arabidopsis thaliana* modified from Finkelstein and Zeevaart (1994) and Sponsel (1995).

elongation is also delayed in *ms33* flowers and auxins are known to stimulate petal growth (Moe, 1971; Zieslin and Halevy, 1976), this too may be related to low auxin in mutant floral buds.

Analysis of endogenous IAA showed that *ms33* flowers contained a low level of IAA as compared to WT (Fig. 34), supporting the view low IAA contributes to inhibition of stamen filament and petal elongation. A reduction in the amount of endogenous IAA was also reported in the male sterile *apetalous* mutant of soybean (Skorupska et al., 1994) and in the *pin-formed* mutant of *Arabidopsis* (Okada et al., 1991).

In normal plants, high levels of auxins have been related to flower development in numerous species. For example, auxin levels increased after flower initiation and during flower development in *Hyoscyamus niger* (Teltscherová et al., 1977) and in *Chenopodium rubrum* (Kopecewicz et al., 1979). Auxin content in the flowering shoots of rose increased during early reproductive development through petal initiation, and was then maintained at this level (Zieslin and Halevy, 1976), or decreased (Moe, 1971). These results further indicate that IAA is required for floral organ growth. Okada et al. (1991) also noted a reduction in the polar IAA transport activity in the *pin-formed* mutant and suggested that the normal auxin polar transport system is required in the early developmental stage of floral bud formation. In some male sterile systems, however, high levels of IAA were observed in leaves and in stamens (Singh et al., 1992; Shukla, 1993). Similarly, in transgenic tobacco, the insertion of a chimeric *rolB* gene, which increases the endogenous IAA level, affected anther and pollen development

(Spena et al., 1992). Based on the analysis of endogenous hormones in the male sterile *apetalous* mutant, Skorupska et al. (1994) suggested that the IAA-ABA ratio may be important in the differential growth and development of the mutant and wild type soybean flowers.

IV.5.3. Abscisic acid

ABA has been reported to induce male sterility in some species, e.g. wheat and rapeseed (Morgan, 1980; Saini and Aspinall, 1982; Shukla and Sawhney, 1994). Exogenous ABA also inhibited stamen development and induced pollen abortion in tomato both *in vitro* (Rastogi and Sawhney, 1988) and *in vivo* (Chandra Sekhar and Sawhney, 1991) as well as countered the GA-promoted development of staminate flowers in cucumber (Rudich and Halevy, 1974; Friedlander et al., 1977). In the present work, *ms33* flowers contained a relatively high level of ABA at normal temperature as compared to WT (Fig. 35). ABA applied to decapitated stamens inhibited filament growth in WT flowers and further suppressed the growth of *ms33* filaments (Table 6). These results suggest that high ABA in mutant flowers is likely a factor in the inhibition of filament growth, delayed petal elongation, and pollen abortion. Similarly, in the male sterile *sl-2* mutant of tomato, high ABA was present in the floral organs as compared to WT and the largest difference was in stamens, the only organ which showed morphological and anatomical abnormalities in mutant flowers (Singh and Sawhney, 1998). High ABA levels were also reported in the stamens of a male sterile mutant in *Brassica napus* (Shukla and Sawhney, 1994). However, male sterility induced by water

stress is not correlated with an increased ABA content (Dembinska et al., 1992; Saini, 1997). It has been further argued that ABA, along with auxins and cytokinins, improve flower development in male sterile soybean (Skorupska et al., 1994) and other species (reviewed by Chailakhyan and Khrianin, 1987). The data from the present work suggests that a high level of ABA, low levels of IAA and GA_4, and/or other growth substances, may be associated with the suppression of stamen filament growth and aberrations in pollen maturation in *ms33* flowers. These results imply that a critical balance of endogenous hormones is required for the growth of floral organs and the expression of male fertility.

IV.5.4. Endogenous hormones in flowers at low temperatures

Temperature is one of the important environmental factors that regulates the expression of male sterility, both in cytoplasmic male sterile systems, e.g. onion (Meer and Bennecom, 1969), *Petunia* (Marrewijk, 1969; Izhar, 1977), cotton (Marshall et al., 1974) and chives (Tatlioglu, 1985), and in genic male sterile systems, e.g. tomato (Rick and Boynton, 1967; Sawhney, 1983b) and Brussels sprouts (Nieuwhof, 1968). It has been suggested that the effect of temperature on stamen and pollen development is mediated through hormonal changes (Singh et al., 1992; Singh and Sawhney, 1998). The effect of low temperature on flower development was also related to high GA activity and to an increase in endogenous levels of GA-like substance (Atherton and Harris, 1980; Sawhney, 1983b; Hazebroek et al., 1993; Ma et al., 1996). The present work showed that in WT flowers, low temperature dramatically reduced the levels of

IAA and GAs (Fig. 33 and 34), but induced a small increase in ABA content; however, male fertility was not affected.

Interestingly, low temperature did not affect the levels of IAA, ABA and GA_3 in *ms33* flowers, but there was an increase in GA_4 concentration. Since low temperature induces partial reversion of male fertility in *ms33* plants, the elevated level of GA_4 may be important for stamen and pollen development. The effect of low temperature on enhanced seed germination, growth of seedlings, and flowering in the *ms33* mutant was also related to an increase in endogenous GA (section III.5.6 and III.5.7). The analysis of endogenous GAs tends to support that suggestion. The increased level of GA_4 establishes a new balance of hormones and it is the altered ratio of hormones which may be responsible for changes in developmental processes. The level of total GAs declined in WT flowers at low temperature as compared to the normal temperature, suggesting that low temperature affects GA biosynthesis or metabolism. However, the ratio of GA_4/GA_3 was higher at low than at normal temperature in WT and *ms33* flowers. This indicates that low temperature favors the formation of GA_4 than GA_3 and further supports the early suggestion that the MS33 product may have a role in GA interconversion.

IV.6. Double mutant analysis

The hormone analysis showed that *ms33* flowers contained relatively high levels of ABA, but low level of total GAs in comparison to WT. Experiments with exogenous hormones showed that GAs enhanced, and ABA suppressed, the growth of stamen

filaments in both the WT and mutant flowers. These observations suggest that the *ms33* mutation likely causes a change in the metabolism of GAs and ABA and/or that it may affect GA and ABA signal transduction pathways. To further determine the role of ABA and GAs in the genetic control of stamen and pollen development, double mutants of *ms33* with an ABA-deficient mutant *aba-1*, and a GA-signal transduction mutant *spy-3* in *Arabidopsis* were constructed

The *aba-1* mutant contains low levels of ABA, and the plants are dwarf, but the flowers are male fertile (Fig. 36, see also Koornneef et al., 1982). In the double mutant *ms33 aba-1*, the phenotype of plants was similar to the *aba-1* mutant, i.e., they were short (Fig. 36C), but the flowers resembled *ms33*, i.e., they had short stamens and produced non-viable pollen (Fig. 36B). Thus, the double mutant showed the additive phenotypic traits of *ms33* and *aba-1*. Since the plant height of the double mutant is similar to *aba-1*, it can be assumed that the ABA level is reduced in the double mutant. However, since male sterility was still expressed in the double mutant, it suggests that the reduction in ABA level alone is not sufficient for the restoration of male fertility in the *ms33* mutant. In the same vein, it can be argued that high ABA content is not the primary factor for the inhibition of stamen growth and pollen abortion in the *ms33* mutant. This is consistent with the conclusion on the water stress-induced male sterility in wheat (Saini, 1997). Further, since ABA level did not significantly change in *ms33* flowers at low temperature, yet male fertility was partially restored, it indicates that hormones other than ABA may be critical for pollen maturation in the *ms33* mutant. A similar suggestion was reported for *sl-2* mutant of tomato in which there was a greater

amount of ABA in stamens, compared to WT (Singh and Sawhney, 1998). Male fertility in *sl-2* can be reversed by low temperature treatment and there was a concomitant drop in the ABA level in stamens, but it was still higher than that in WT stamens (Singh and Sawhney, 1998). On the other hand, male fertility in *sl-2* can be restored by application of GA_3 (Sawhney and Greyson, 1973). This supports the view that more than one hormone is likely involved in the expression of male sterility.

Physiological functions of plant hormones are determined either by changes in their relative concentrations, i.e., through the biosynthesis or metabolism, or by hormonal perception and signal transduction. Some GA signal transduction mutants (known as 'slender' mutants) have been isolated from barley (Foster, 1977), tomato (Jones, 1987) and *Arabidopsis* (Jacobsen and Olszewski, 1993). These mutants exhibit the phenotype similar to that of WT plants treated with high doses of GA (Foster, 1977; Jacobsen and Olszewski, 1993), i.e., long internodes, early flowering and partial or complete male sterility. These mutants also contained lower levels of GAs than their WT plants (Potts et al., 1985; Jones, 1987; Croker et al., 1990). The question whether GA signal transduction is affected in the *ms33* mutant was addressed by constructing a double mutant of *ms33* with a GA signal transduction mutant *spy-3*.

spy-3 mutant has the same phenotype as the slender mutants, i.e., tall plants, early flowering, but is partially male sterile (Fig. 37, see also Jacobsen and Olszewski, 1993). In the double mutant *ms33 spy-3*, the phenotype of plants was similar to *spy-3*, i.e., long peduncles and early flowering and flowers were male sterile. The measurements of floral organs showed that the size of double mutant flowers was larger

than either of the single mutants. Also, whereas the stamen length was increased in *ms33 spy-3*, it was still shorter than the gynoecial length (Table 9), and pollen produced were non-viable. Thus, the phenotype of the double mutant flowers was, in principle, similar to that of *ms33*. These results indicate that the stimulation of the GA signaling pathway by *spy-3* does not overcome the relative inhibition of stamen filament growth, and the abortion of pollen development in *ms33* mutant. The observations also suggest that in the *ms33* mutant the expression of male sterility is unrelated to a possible blockage of the GA signal transduction pathway. As discussed earlier, the *ms33* mutation likely causes a defect in GA biosynthesis, or GA interconversion. When the male sterile double mutant plants were grown in low temperature (15/11°C), a partial reversion of male fertility was obtained, which was similar to *ms33* grown in the same conditions. This further suggests that low temperature effects on *ms33* are also not related to GA signaling, but rather to GA biosynthesis, or interconversion.

Taken together the results presented here suggest that low level of ABA and a high level of total GAs is not required for pollen maturation in *Arabidopsis*, but an elevated level of GA_4 is likely critical. However, for stamen filament growth the increased levels of GAs seems to be important. A high level of GA_4 and/or a critical balance of GA_4 with other hormones may be required for the complete restoration of male fertility in the *ms33* mutant.

V. SUMMARY AND CONCLUSIONS

Morphological characterization of the single recessive *male sterile33* (*ms33*) mutant in *Arabidopsis* revealed that the major defects in the flowers were inhibition of the rapid stamen filament growth just before anthesis, and the production of non-viable pollen. However, the *ms33* mutation also caused several pleiotropic effects, including aberrant growth of all floral organs, and delayed seed germination, seedling and flowering time.

Ultrastructural studies on stamen and pollen development showed that the *ms33* mutation induces premature breakdown of the tapetal tissue and this had an effect on the deposition of tryphine on the pollen wall, the prematuration of the intine, and reduced amount of reserve (lipid bodies) in the pollen cytoplasm. In addition, the mutant pollen grains were of large size and had large vacuoles, compared to WT pollen, indicating the lack of, or reduced, pollen desiccation. Since dehydration is an important component of pollen maturation, non-desiccation of *ms33* pollen likely affects its viability.

Observations on filament growth indicated that the *ms33* mutation results in the suppression of rapid growth before anthesis, and this is related to retardation of cell elongation. Thus, the *MS33* gene is required for enhancing cell elongation and filament growth before anthesis. Experiments with exogenous hormones showed that GAs or IAA can overcome the inhibition, but that ABA or ethrel further suppress growth. This suggests that in the mutant there is an imbalance of plant growth substances, i.e., low

level of promoters (e.g. GAs and IAA) and/or high level of inhibitors (e.g. ABA) supplied mainly by the anther as well as other floral tissues. These results also suggested that the MS33 product may regulate a temporal increase in the biosynthesis of GA and IAA, or stimulate the GA or IAA signal transduction pathway which in turn induces cell elongation in the filament.

Seed germination experiments showed that *Arabidopsis* seed is light-requiring, and the germination is presumably regulated by phytochrome and that its action is likely mediated through GAs. Low germination in the *ms33* mutant may be associated mainly with low levels of GAs, not with the activity of phytochromes. GA_4 was more effective than GA_3 in inducing seed germination, and seedling and plant growth in the *ms33* mutant.

Analyses of endogenous hormones showed that WT flowers contain higher levels of total GAs than the *ms33* mutant, and the strikingly high level of GA_4 in WT flowers indicates that GA_4 is the major GA in *Arabidopsis*. Mutant flowers contained low level of GA_4, but had high GA_3 content. Since GA_4 is more effective than GA_3 on the growth of *Arabidopsis* plants, it is suggested that the *ms33* mutation causes a shift in favor of the early 13-hydroxylation pathway instead of the non-13-hydroxylation pathway. WT flowers also contained a higher level of IAA, and lower ABA than *ms33*. The hormone levels at low temperature showed that in WT flowers GA and IAA levels declined, and ABA increased, but male fertility was not affected. In the *ms33* flowers the levels of IAA and ABA were not reduced, but GA_4 increased. Moreover, a partial restoration of male fertility is obtained in the *ms33* mutant grown at low temperature.

These results suggest that the partial reversion of male fertility in the *ms33* mutant by low temperature may be associated with increase in GA_4 level, and that this establishes a new balance with other hormones.

The construction of double mutants of *ms33* with an ABA deficient mutant *aba-1*, and a GA-signal transduction mutant *spy-3*, revealed that the inhibition of stamen filament growth and loss of pollen viability in the *ms33* mutant is unrelated to either high ABA content or blockage in the GA signal transduction pathway.

In conclusion, mutation in the *MS33* gene causes several pleiotropic effects from seed germination to pollen maturation. Many of these defects can be explained on the basis of reduced levels of total GAs, and in particular GA_4. The data suggest that in the *ms33* mutant the early 13-hydroxylation pathway of GA biosynthesis is activated, resulting in the accumulation of GA_3. In the WT *Arabidopsis*, the non-hydroxylation pathway is favored, which results in greater levels of GA_{15}, GA_{24}, GA_9 and GA_4.

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